

IN VITRO ANALYSES OF PROTEIN TRANSPORT INTO THYLAKOIDS:
A SUBSET OF CHLOROPLAST PROTEINS ARE TRANSPORTED INTO
THYLAKOIDS BY A CHLOROPLAST *SECA*-DEPENDENT PATHWAY

By

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KEY TO ABBREVIATIONS

| | |
|--------------|---|
| LHCP | Light-harvesting chlorophyll a/b protein |
| PC | plastocyanin |
| PCara | plastocyanin from <i>Arabidopsis thaliana</i> |
| PCpea | plastocyanin from pea |
| OE33 | the 33-kDa subunit of the oxygen-evolving complex |
| OE23 | the 23-kDa subunit of the oxygen-evolving complex |
| OE17 | the 17-kDa subunit of the oxygen-evolving complex |
| Rubisco | ribulose-1,5-bisphosphate carboxylase/oxygenase |
| SS | small subunit of Rubisco |
| pLHCP | precursor form of LHCP |
| pPC | precursor form of PC |
| pOE33 | precursor form of OE33 |
| pOE23 | precursor form of OE23 |
| pOE17 | precursor form of OE17 |
| pSS | precursor form of SS |
| (p)LHCP | pLHCP or LHCP |
| LHCP-DP | a characteristic protease degradation product of LHCP |
| mPC | mature form of PC |
| mOE33 | mature form of OE33 |
| mOE23 | mature form of OE23 |
| mOE17 | mature form of OE17 |
| SE | stromal extract |
| BSA | bovine serum albumin |
| Hsp70 | 70-kDa heat shock protein |
| DTT | dithiothreitol |
| PMF | proton motive force |
| $\Delta\psi$ | transmembrane electric potential |
| E | einstein |
| CF_1/CF_0 | coupling factor |

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Thylakoids are membranes within chloroplasts that carry out the light-driven reactions of photosynthesis. Biogenesis of thylakoids requires the cooperative expression of both the chloroplast genome and the nuclear genome. Nuclear encoded thylakoid proteins are synthesized in the cytosol, imported into chloroplasts, and then transported into thylakoids. To understand the mechanisms for protein targeting to the thylakoids, *in vitro* assays with isolated chloroplasts and isolated thylakoids were developed and used for the studies of thylakoid protein transport.

In the first part of this dissertation, a method for preservation of isolated chloroplasts and isolated thylakoids is presented to facilitate investigations of thylakoid protein biogenesis.

In the second part of this dissertation, a purified thylakoid membrane precursor protein was used in *in vitro* assays to investigate the importance of soluble factors in protein import into chloroplasts and integration into thylakoids. The results show that cytosolic factors are not required for import into the chloroplast stroma, but integration into the thylakoid membrane absolutely depends on a stromal protein factor that appears to play an active role in the integration process. Purified thylakoid precursor proteins were also used in substrate competition studies that revealed three distinct targeting pathways for transport of proteins into or across the thylakoid membrane.

In the third part of this dissertation, *in vitro* assays with isolated thylakoids were developed for detailed studies on the requirements for transport of proteins on one of the pathways. The results show that similar requirements are used for transport of proteins on the same translocation pathway. Further studies on the properties of this thylakoid translocation pathway show that it exhibits characteristics of SecA-dependent protein export from bacteria, suggesting a SecA-homologous pathway for protein transport into plant thylakoids. With an antibody to a conserved peptide from an algal SecA, a SecA homologue of pea chloroplasts (CPSecA) was identified and purified from stromal extract with a combination of conventional purification methods. Purified CPSecA was shown to be able to support protein transport into isolated thylakoids. Interestingly, only one of the three pathways for protein transport into thylakoids uses the chloroplast SecA-dependent translocation mechanism.

CHAPTER 1 INTRODUCTION

Chloroplasts make this planet green. They are the chlorophyll-containing organelles in plant cells that are responsible for the conversion of light energy into chemical energy in photosynthesis. Besides being the organelle of photosynthesis, chloroplasts are also the site of many other biosynthetic functions such as synthesis of amino acids, lipids, and pigments (Kirk and Tilney-Bassett, 1978). All these functions are catalyzed by enzymes made up of proteins. Although chloroplasts have their own machinery for making proteins, less than 15% of their proteins are made in the chloroplast. The majority of chloroplast proteins are encoded in the nucleus, synthesized in the cytosol, and posttranslationally imported into chloroplasts. The mechanisms that govern protein targeting and transport are thus of exceptional importance to the biogenesis of chloroplasts. However, despite intensive studies over the last two decades, still very little is known about the mechanisms by which nuclear-encoded chloroplast proteins are specifically targeted to the six chloroplast subcompartments [see de Boer and Weisbeek (1991) and Theg and Scott (1993) for a recent reviews].

Goals of the Research

The overall goal of our research presented here is to understand the strategies and mechanisms by which newly made chloroplast proteins are specifically localized to the various subcompartments. As an experimental system we have chosen as a model the targeting and transport of cytosolically-synthesized chloroplast proteins to the thylakoids because they have the most complex pathway for localization. They have to cross up to three membranes and three aqueous compartments *en route* from their site of synthesis in the cytosol to their functional locations in the thylakoid membrane or the lumen. Biochemical studies of this process have made use of two *in vitro* assays for the assessment of import into chloroplasts and transport into thylakoids. The first is called the import assay that utilizes isolated intact chloroplasts and radiolabeled precursors. The second is called the integration or transport assay that is similar to the import assay with the exception that it employs lysed chloroplasts and isolated thylakoids supplemented with stromal extract. To facilitate such studies, the first specific goal of this dissertation is to develop a method for preservation of active chloroplasts and thylakoids for studies of protein import and integration. A second specific goal of this dissertation is to produce chemical quantities of pure precursor proteins that can be used to address questions about the importance of soluble factors in chloroplast protein transport and to carry out substrate competition studies that could reveal pathway specificity for import into chloroplasts or transport into thylakoids. The third specific goal of this dissertation is to purify a stromal component required for transport of a subset of proteins into

the thylakoid lumen. The results from these studies are presented in four chapters (Chapters 2-5). The purpose of this chapter is to give some background information and to outline the questions related to the studies described in this dissertation. The results and significance are summarized and discussed in the last chapter.

Structure and Function of Chloroplasts

Chloroplasts are the photosynthetic member of a family of organelles called plastids that distinguish plants from other biological kingdoms. Chloroplasts are found in leaves and other green tissues of plants. Chloroplasts are structurally complex organelles. They are enclosed by a double membrane envelope and are filled with an internal membrane system called the thylakoids. The envelope membranes and the thylakoid membrane divide chloroplasts into several subcompartments. The intermembrane space is the aqueous compartment between the two envelope membranes. The thylakoid lumen is the aqueous compartment enclosed by the thylakoid membrane. The stroma is the aqueous compartment within the envelope and outside the thylakoids.

The chloroplast envelope separates the contents of the chloroplast from the cytoplasm of the cell. The major role of the envelope is to serve as a selective barrier and to allow regulated exchange of metabolites and ions between chloroplasts and the cell cytosol (Douce and Joyard, 1991). The envelope also contains an import machinery for transport of proteins into the chloroplast (Douce and Joyard, 1990). In addition to its many transport functions, the envelope is also the site of many

biosynthetic reactions such as synthesis of lipids and pigments (Douce et al., 1984; Douce and Joyard, 1991). The stroma is the site of dark reactions of photosynthesis, the site of protein synthesis from chloroplast-encoded genes, and the site of many other reactions. The thylakoids are the site of the light reactions of photosynthesis. In the light reactions of photosynthesis, light energy is captured by the two photosystems and used to drive electron transfer that results in the production of NADPH and the build-up of a proton gradient across the thylakoid membrane. The proton gradient is subsequently used by the thylakoidal ATP synthase to make ATP. The thylakoid membrane contains the lipids, the pigments and most of the proteins involved in the light reactions of photosynthesis. The only components of the light reactions that do not permanently associate with the membrane are the electron carriers plastocyanin and ferredoxin which, respectively, reside in the thylakoid lumen and the chloroplast stroma (Bogorad, 1991).

Biogenesis of Chloroplasts

Different forms of plastids, each with distinct functions, are found in plants. These different forms are all referred to as 'plastids' because they are all, to some extent, interconvertible (Kirk and Tilney-Bassett, 1978). The best-known plastid is the chloroplast. Other plastids include proplastids, etioplasts, amyloplasts, and chromoplasts. Proplastids are undifferentiated progenitor plastids present in meristematic cells. All other plastids ultimately develop from proplastids by division and differentiation (Mullet, 1988). Proplastids are small and contain little thylakoid

membrane system, but during development they increase in size, accumulate numerous macromolecular components, and progressively deposit membrane lamellae in their stroma. In leaves, the end result of proplastid development is chloroplast with its highly intricate internal membrane system. The development of chloroplasts from their tiny precursor proplastids involves synthesis of a great deal of lipids, pigments, nucleic acids, and proteins.

Synthesis of the fatty acids occurs in the chloroplast stroma (Slabas and Fawcett, 1992) along with the initial acylation of glycerol 3-phosphate (Alban et al., 1988; Browse and Sommerville, 1991). The later steps of lipid synthesis are carried out by enzymes in/on the envelope membranes (Joyard et al., 1991). The lipid of the thylakoid membrane is thought to be transferred from the envelope as membrane vesicles (Joyard et al., 1986). Because protein is the major component of chloroplasts, chloroplast development involves a massive synthesis of proteins. Chloroplasts contain DNA and have a machinery for protein synthesis. However, the capacity of the chloroplast genome is too small to account for all the proteins present in the chloroplast (Palmer, 1991). Indeed, the majority of the chloroplast proteins are encoded in the nucleus, synthesized in the cytosol, and transported into chloroplasts. Nuclear-encoded chloroplast proteins are first synthesized as larger precursors with amino terminal extensions called transit peptides that are both necessary and sufficient for directing import into the chloroplast (Berry-Lowe and Schmidt, 1991). Some multimeric enzymes and protein complexes of chloroplasts are composed of both chloroplast- and nuclear-encoded components. An interesting but

also very important question about chloroplast biogenesis is how the cells coordinate the expression of chloroplast- and nuclear-encoded proteins destined for the same functional complex and how proteins are specifically targeted to their correct location in the chloroplast. The targeting and transport of proteins to the chloroplast and their subsequent localization within the chloroplast are the focuses of the research described in this dissertation.

Protein Import into Chloroplasts

A key development that allowed significant advances in our understanding of protein import into chloroplasts was the reconstitution of protein transport with isolated chloroplasts (Chua and Schmidt, 1978, 1979; Highfield and Ellis, 1978). Studies using reconstituted system have shown that protein import into chloroplasts is a multi-step process. The first step is the binding of precursor proteins to the chloroplast surface (Cline et al., 1985; Su et al., 1992). The second step is translocation of the bound precursors across the two envelope membranes into the stroma (Schnell and Blobel, 1993). A third step, which occurs during or shortly after translocation, is proteolytic processing of the precursor to remove the transit peptide (Berry-Lowe and Schmidt, 1991). *In vitro* assays for import studies depend on the availability of isolated intact chloroplasts. A method for long-term preservation of intact active chloroplasts would be helpful for such studies.

Structure of Chloroplast Transit Peptides

Most chloroplast precursor proteins possess monopartite transit peptides for targeting to the chloroplast stroma. These transit peptides from different precursor proteins are functionally interchangeable (Berry-Lowe and Schmidt, 1991; de Boer and Weisbeek, 1991). However, there is no apparent sequence homology between transit peptides of different precursor proteins. Transit peptides are characterized by large numbers of serine and threonine and the lack of tyrosine and negatively charged amino acids (Berry-Lowe and Schmidt, 1991). Transit peptides have been predicted to form random coils (von Heijne and Nishikawa, 1991). Theg and Geske (1992) have investigated the biophysical properties of the transit peptide to the gamma subunit of the chloroplast ATP synthase from *Chlamydomonas reinhardtii*. They used circular dichroism measurements to show that the gamma subunit transit peptide is largely a random coil, both in the presence and in the absence of sodium laurylsulfate micelles. However, the secondary structure of the ferredoxin transit peptide was reported to be modulated by its interaction with negatively charged lipids (Horniak et al., 1993). In buffer, the transit peptide of ferredoxin was shown to be in a random coil conformation. In the presence of micelles formed by anionic lipids, a large increase in α -helix was observed for the ferredoxin transit peptide. Transit peptides are removed in the stroma by a soluble processing protease (de Boer and Weisbeek, 1991; Berry-Lowe and Schmidt, 1991).

Energetics of Protein Import into Chloroplasts

Import of proteins into chloroplasts is an energy-dependent process (Grossman et al., 1980; Pain and Blobel, 1987). Unlike the import of proteins into mitochondria which requires the presence of both ATP and an electric field across the inner mitochondrial membrane (Chen and Douglas, 1987; Eiler et al., 1987), import of proteins into chloroplasts needs only ATP (Theg et al., 1989). ATP is required both for efficient binding of precursor proteins to the chloroplast envelope and for translocation of bound precursors across the two envelope membranes into the stroma. Binding requires only micromolar concentrations of ATP (Olsen et al., 1989). The site of ATP utilization for binding is the intermembrane space between the two envelope membranes (Olsen and Keegstra, 1992). The ATP requirement for protein transport across the envelope is about tenfold higher than that for precursor binding to the chloroplast surface (Olsen et al., 1989; Theg et al., 1989). The site of ATP hydrolysis for import is in the stroma (Pain and Blobel, 1987; Theg et al., 1989).

Cytosolic Factors for Protein Import into Chloroplasts

Over the past several years, a number of studies have used purified precursor proteins to investigate the requirement for cytosolic factors for protein import into chloroplasts. Chemical quantities of purified precursor proteins were obtained by overexpression in *E. coli* and then purification from cell extract by conventional chromatography or by isolation of inclusion bodies. Purified precursor proteins were dissolved in 8 M urea and assayed for import in the presence or absence of cytosolic

proteins immediately after dilution from urea. When the precursor protein to the light-harvesting chlorophyll a/b protein (pLHCP) was used in such studies, no import was obtained after urea-denatured pLHCP was rapidly diluted into the import mixture (Waegemann *et al.*, 1990). Import of pLHCP into isolated chloroplasts was only observed after the denatured pLHCP was dialyzed in the presence of leaf extract. The effect of the leaf extract on the import of pLHCP into chloroplasts was only partly compensated by Hsp70. Based on these experiments, the authors concluded that import of proteins into chloroplasts requires at least two cytosolic factors. On the other hand, efficient import was obtained for purified precursors of plastocyanin (de Boer *et al.*, 1991) and ferredoxin (Pilon *et al.*, 1990) in the absence of cytosolic factors, indicating that cytosolic factors are not necessary for protein import into chloroplasts. Apparently, there is a difference between the import requirement for pLHCP and that for pre-ferredoxin and pre-plastocyanin. Does this difference in import requirements reflect the difference between precursor proteins or between the apparatus for import of pLHCP and that for import of pre-ferredoxin or pre-plastocyanin? If the latter were true, it would mean that different mechanisms were used for the import of specific proteins into the chloroplast. As will be seen in chapter 3, our studies challenge the conclusions of Waegemann *et al.* (1990).

Membrane Proteins Involved in Chloroplast Protein Import

Before import into chloroplasts, precursors must bind to the envelope of chloroplast. Binding has been shown to be specific and saturable (Cline et al., 1985; Friedman and Keegstra, 1989). Binding was greatly reduced when chloroplasts were treated with protease (Cline et al., 1985). These results suggest that binding of precursor proteins to the chloroplast envelope is mediated by proteinaceous receptors, consistent with the fact that high affinity binding requires low levels of ATP. In an effort to identify the import receptor(s), Cornwell and Keegstra (1987) used a noncleavable heterobifunctional crosslinker and crosslinked a putative 66-kDa receptor protein to a radiolabeled small subunit of ribulose-1,5-bisphosphate carboxylase precursor (pSS). However, they were unable to identify a 66-kDa protein on stained gels of purified envelope proteins.

Kaderbhai et al. (1988) performed cross-linking to isolated inner and outer envelope membranes using a synthetic peptide corresponding to the transit peptide of pSS and identified a 30-kDa and a 52-kDa protein. Pain et al. (1988) used antiidiotypic antibodies and also identified a 30-kDa and a 52-kDa protein. The 52-kDa protein was shown to be the large subunit of ribulose bisphosphate carboxylase. The 30-kDa protein was found by sequencing of a cDNA clone to be the same protein identified previously as the phosphate translocator (Schnell et al., 1990; Flugge et al., 1991; Wiley et al., 1991).

A 51-kDa protein of possible involvement in chloroplast protein import was identified by yet another approach that used ^{32}P -ATP to examine differential

phosphorylation of envelope proteins upon interaction with precursor proteins (Hinz and Flugge, 1988). More recently, an 86-kDa protein, a 34-kDa protein, and a 70-kDa heat shock cognate protein were identified and shown to be in a complex that could interact with precursor proteins in an ATP-dependent manner (Waegemann and Soll, 1991; Soll and Waegemann, 1992; Soll and Alesen, 1993).

Most recently, Perry and Keegstra (1994) performed label transfer crosslinking experiments and labeled two proteins of 75-kDa and 86-kDa of potential involvement in chloroplast protein import. Labeling of both proteins required pSS with its transit peptide. Labeling of the 75-kDa protein occurred only when low levels of ATP were present. Both proteins were identified as proteins of the outer envelope membrane. However, the labeled form of the 75-kDa protein could only be detected in fractions containing mixed envelope membranes. These results suggest that pSS first binds to the 86-kDa protein and that the ATP-requiring step for binding is associated with the 75-kDa protein.

Protein Transport into Thylakoids

Nuclear-encoded proteins destined for the internal membrane system of chloroplasts must be further transported into or across the thylakoid membrane in order to reach their functional locations. Studies with a number of different thylakoid proteins (Smeekens et al., 1986; Ko and Cashmore, 1989; Cline et al., 1993; Howe and Merchant, 1993) have indicated that thylakoid precursor proteins are first translocated across both envelope membranes into the chloroplast stroma, and are

then transported into the thylakoid membrane or across the membrane into the lumen.

Signals for Localization to the Thylakoids

In addition to the signals for envelope transfer, precursors of thylakoid luminal proteins also contain a sequence for thylakoid transfer on their amino terminal extensions. The amino terminal extensions of luminal proteins are thus bipartite in structure with a stroma-targeting transit peptide at the N-terminus proximal and a lumen-targeting sequence for thylakoid transfer at the C-terminus proximal (de Boer and Weisbeek, 1991). Lumen-targeting sequences are structurally similar to signal peptides of bacterial and endoplasmic reticulum (ER) secretory proteins. They are all characterized by having a hydrophobic core region of ~ 15 residues, a positively charged N-flanking region and an A-X-A cleavage site (von Heijne et al., 1989). Lumen-targeting sequences are removed in the lumen by a thylakoidal peptidase (Halpin et al., 1989).

There is no cleavable signal sequence for the integration of proteins into the thylakoid membrane. Integration of membrane proteins such as the light-harvesting chlorophyll a/b-binding protein of photosystem 2 (LHCP) is directed by targeting signals within the mature protein sequence (Lamppa, 1988; Viitanen *et al.*, 1988; Hand et al., 1989). The exact location of these signals in LHCP has not been determined, but appears to lie within one or more of the three hydrophobic transmembrane segments (Auchincloss *et al.*, 1992; Huang et al., 1992).

Pathways for Protein Transport into Thylakoids

Several lines of evidence suggest that a common translocation mechanism is used for the transport of proteins across the envelope membranes. The most convincing evidence comes from competition studies. Purified precursor proteins and synthetic transit peptides compete for the import of other precursor proteins (Perry et al., 1991; Schnell et al., 1991; Oblong and Lamppa, 1992; Cline et al., 1993). Import of both stromal and thylakoid precursor proteins was severely inhibited by import saturating concentrations of pOE23 (Cline et al., 1993).

In contrast to protein transport across the envelope into the stroma, transport of proteins into thylakoids appears to be mediated by several distinct translocation mechanisms. Evidence for the operation of multiple pathways for protein transport into thylakoids comes from recent substrate competition studies that revealed three distinct precursor specificity groups (Cline et al., 1993). Lumen-resident proteins OE23 and OE17 constitute one group, luminal proteins OE33 and PC a second, and the membrane protein LHCP a third. Two additional proteins PS1-N and PS2-T were subsequently shown to be on the OE23 and OE17 pathway (Henry et al., 1994; Nielsen et al., 1994). The specificity group determined from competition studies correlate exactly with the precursor protein-specific requirements for transport of proteins into thylakoids (Cline et al., 1992a). However, it remains to be seen whether the various pathways represent distinct translocation systems.

Energetics of Protein Transport into Thylakoids

In contrast to the singular ATP requirement for import of proteins into chloroplasts, transport of proteins into thylakoids requires both nucleotide triphosphates and a proton motive force (PMF) (Mould and Robinson, 1991; Cline et al., 1989, 1992; Reed et al., 1990). The ΔpH of the PMF is the active component for thylakoid protein transport. The energy requirements for thylakoid protein transport are further complicated by the fact that the energy requirements for thylakoid transport are precursor protein-specific (Cline et al., 1992). Transport of the 33-kDa subunit of the oxygen-evolving complex (OE33) and integration of LHCP requires ATP and is stimulated by the trans-thylakoidal proton gradient (Kirwin et al., 1989; Bauerle and Keegstra, 1991; Mould and Robinson, 1991; Cline et al., 1992). Transport of plastocyanin (PC) requires only ATP (Theg et al., 1989; Cline et al., 1992). Transport of OE23 and OE17 (the 23-kDa and 17-kDa subunit of the oxygen-evolving complex) require only a proton gradient (Cline et al., 1992). Why are there different energy requirements for protein transport into thylakoids? Does this reflect different mechanisms for thylakoid transport?

Soluble Factors Required for Thylakoid Transport

The stromal factor required for integration of LHCP into the thylakoid membrane is the first soluble factor ever reported for chloroplast protein transport (Fulsom and Cline, 1988). This factor was originally discovered during *in vitro* integration studies with separated chloroplast subfractions (Cline, 1986). It was

found that membrane integration of pLHCP occurred only in the presence of both thylakoids and stromal extract. The stromal factor has been shown to be a protein factor with an active sulfhydryl group (Fulsom and Cline, 1988). Payan and Cline (1991) have shown that one function of the stromal factor is to maintain the solubility and insertion competence of LHCP by converting LHCP into a large and soluble species. Efforts to identify and isolate this factor have already been undertaken in our laboratory. Purification was performed with conventional protein fractionation methods based on integration activity. Unfortunately, integration activity was lost when the resolution of separation methods was increased. Stromal factor was also reported for efficient transport of OE33 into the thylakoid lumen (Mould et al., 1991). It was suggested that the stromal processing activity was the stromal stimulating activity for OE33 transport (Kirwin et al., 1989; Mould et al. 1991). However, their experiments could not rule out the possibility that a stromal factor other than the processing protease is the stimulating activity for OE33 transport.

Protein Export from Bacteria as a Model for Studies of Protein Transport into Thylakoids

The evolutionary ancestor of chloroplasts is thought to be a cyanobacterium that formed an endosymbiotic existence with the host primordia eukaryote. Similarly, the evolutionary ancestor of the mitochondrion is thought to be an endosymbiotic purple bacterium. In this context, there is good reason to believe that the protein sorting and/or transport system that occurs within chloroplasts and mitochondria is

derived from the machinery present in the endosymbiotic prokaryotes at the time of endosymbiosis. This concept has been referred to as "Conservative Sorting" in the mitochondrial literature (Hartl and Neupert, 1990). The "Conservative Sorting" hypothesis suggests that intraorganellar protein transport systems resemble those of modern-day prokaryotes. The mechanisms for thylakoid protein transport might also be very similar to the transport of proteins across the bacterial membrane (Smeekens et al., 1990). Indeed, there is much similarity between thylakoid protein transport and protein export in bacteria. First, signal sequences for thylakoid transport are like bacterial signal peptides. Second, the thylakoid transfer sequences have been shown to function in *E. coli* as signal peptides and are able to export the passenger protein from the cytoplasm. Third, the thylakoidal peptidase that removes the thylakoid transfer domain has identical substrate specificity as that of the bacterial signal peptidase that removes signal peptides from bacterial secretory proteins (de Boer and Weisbeek, 1991; Berry-Lowe and Schmidt, 1991; and references within).

The General Secretory Pathway in Bacteria

Currently, the best understood system for protein export from bacteria is that of *E. coli*. In *E. coli*, signal sequence-bearing proteins are exported by a translocation system that depends on SecA and SecY/E proteins and is powered by the combined action of ATP hydrolysis and a proton motive force (Pugsley, 1993). Some preproteins also require SecB for efficient export (Kumamoto and Bechwith, 1983, 1985). Two additional proteins SecD and SecF are essential for protein export

in vivo (Gardel et al., 1990), but have no effect *in vitro* (Matsuyama et al., 1993). SecA and SecB are found in the cytosol, although SecA is also found associated with the cytoplasmic membrane (Oliver, 1993). SecY, SecE, SecD and SecF are membrane proteins. SecY seems to span the membrane 10 times with its amino and carboxyl termini in the cytosol (Ito, 1992). SecE from *E. coli* is thought to span the membrane three times, although the *Bacillus subtilis* SecE only spans the membrane one time (Schatz et al., 1989; Ito, 1992). SecD and SecF both appear to span the membrane six times (Gardel et al., 1990).

SecB is a chaperone protein that binds to precursor proteins and maintains them in an export competent conformation. SecB also helps targeting precursor proteins to the translocase (an enzyme made up of SecA and SecY/E proteins) via its affinity for SecA (Hartl et al., 1990). SecA is the peripheral domain of the protein translocase. SecA hydrolyzes ATP and initiates translocation by getting the first 20 amino acid residues across the *E. coli* membrane (Schiebel et al., 1991). In the absence of a PMF, SecA and ATP can complete translocation by promoting transport of ~20 amino acid residues each cycle across the membrane (Schiebel et al., 1991). Azide inhibits SecA-dependent transport (Oliver et al., 1990) and removal of membrane-associated SecA by urea extraction prevents transport (Cunningham et al., 1989). Addition of either purified SecA or SecA-containing fraction restores transport activity to urea-washed membranes. SecY and SecE may form a pore for the actual passage of preproteins across the membrane (Joly and Wickner, 1993).

The functions of SecD and SecF are not yet clear, but recent studies suggest that they may be involved in the PMF-driven transport (Arkowitz and Wickner, 1994).

Is Protein Transport into Thylakoids Homologous to Protein Export from Bacteria?

The endosymbiotic origin of chloroplasts from an ancestor cyanobacterium, the similarity between the thylakoid targeting sequences and the bacterial signal sequences, the identical cleavage specificity of the thylakoidal and bacterial peptidases, the discovery of *secA* homologous genes in the chloroplast genomes of several algae all point to the existence of a SecA type of protein translocation system in plant chloroplasts. However, direct evidence for the operation of a SecA type of translocation mechanism for protein transport into plant thylakoids must come from the identification of a chloroplast homologue of SecA and the demonstration of its involvement in protein transport into thylakoids.

CHAPTER 2

CRYOPRESERVATION OF CHLOROPLASTS AND THYLAKOIDS FOR STUDIES OF PROTEIN IMPORT AND INTEGRATION

Introduction

Recent advances in our understanding of the mechanisms of chloroplast protein biogenesis derive primarily from the availability of *in vitro* biochemical assays for chloroplast protein import and *in organello* protein synthesis. Active chloroplasts are prepared for these assays by homogenization of fresh tissue followed by a combination of differential and density gradient centrifugation (Walker et al., 1987). Chloroplasts are then incubated under conditions that either promote synthesis of proteins within the organelle (Bhaya and Jagendorf, 1984; Mullet et al., 1986) or allow the import of cytoplasmically synthesized plastid proteins into the organelle (Mishkind et al., 1987). Isolation of chloroplasts from amenable plant species, such as spinach and peas, is not difficult but requires about two intensive hours from start to finish. This preparation time has become a limiting factor as investigations of transport mechanisms have become more sophisticated and the duration of experiments has increased. A ready source of preserved but active chloroplasts would greatly facilitate such studies. Furthermore, the ability to preserve active chloroplasts for extended periods will obviate the problem of seasonal variation in

import competence of chloroplasts and permit investigators to comparatively analyze plastids isolated from different plant tissue at different times.

Recent studies of plastid protein biogenesis have also focused on stromal factors necessary for assembly of thylakoid proteins (Chitnis et al., 1987; Cline, 1986; Fulsom and Cline, 1988). Purification schemes for the stromal factors require reconstitution with active thylakoids for activity assays. Such assays are frequently conducted one or several days after the original isolation of stroma and require the isolation of fresh chloroplasts for thylakoid preparation. Accordingly, it would be very helpful if thylakoids from the original chloroplast preparation could be frozen for later use.

Methods for preservation of photosynthetically competent thylakoids have been available for several years (Farkas and Malkin, 1979; Santarius, 1986; 1990), but until now, methods for preserving intact chloroplasts have not been described. The present communication describes protocols for the preservation of intact chloroplasts and isolated thylakoids that are, respectively, active for subsequent studies of protein import and integration. Approximately 65-70% of the chloroplasts stored in liquid nitrogen in the presence of dimethyl sulfoxide remained intact upon thawing and were fully functional for the import of precursor proteins. Preserved thylakoids were nearly as active for protein integration studies as freshly prepared thylakoids. The ability to store chloroplasts and subfractions for extended periods will facilitate investigations of plastid protein biogenesis.

Materials and Methods

Materials

Tritium-labeled leucine was purchased from New England Nuclear. RNasin and SP6 polymerase were from Promega Biotech. Miracloth was from Behring Diagnostics. Mg-ATP, thermolysin, and Percoll were from Sigma. DMSO was from Aldrich Chemical Company. Ethylene glycol and glycerol were from Fisher Chemical Company. All other chemicals were reagent grade. The *in vitro* expression plasmid for pLHCP, psAB80XD/4, is an SP6 derivative of psAB80 (Cashmore, 1984) and has been described elsewhere (Cline et al., 1989). The expression plasmid for LHCP, P2HPLC, is a pUC18 plasmid that harbors the coding sequence for the mature form of LHCP from petunia (Viitanen et al., 1988), and was generously provided by Dr. Paul Viitanen. The expression plasmid for the precursor to the Rubisco small subunit from pea, pSMS64 (Anderson and Smith, 1986), was the generous gift of Dr. Steven Smith. The expression plasmid for the precursor to plastocyanin, pSPPC74 (Smeekens et al., 1985), was kindly provided by Drs. Thomas Lubben and Kenneth Keegstra.

Preparation of Chloroplasts, Lysates, Thylakoids, and Stromal Extract

Intact chloroplasts were isolated from 10- to 12-day-old pea (Laxton's Progress 9) seedlings as described (Cline, 1986). Chloroplast lysates were obtained from intact chloroplasts by resuspending chloroplast pellets in 10 mM Hepes/KOH (pH 8), 10 mM MgCl₂, and after 5 min adjusting to import buffer, 10 mM MgCl₂.

Thylakoids were prepared from chloroplast lysates by centrifugation at 3200 x g for 8 min at 4 °C. Stromal extract was prepared from the resulting supernatant by further centrifugation at 42,000 x g for 30 min at 4°C. Lysates prepared at a concentration of 0.5 mg Chlorophyll/mL are arbitrarily referred to as 1X lysate and the stromal extract resulting from such lysates as 1X stroma. Chlorophyll (Chl) concentrations were determined according to Arnon (1949). For cryopreservation, intact chloroplasts were resuspended in import buffer containing varying amounts of cryoprotectant (0, 10, 20, or 30% [v/v] DMSO, ethylene glycol, glycerol; or 0.1 M, 0.2 M, or 0.3 M glycine) at final concentrations of 3.0 - 5.0 mg Chl/mL. Thylakoids to be cryopreserved were resuspended in storage buffer (20 mM Hepes/KOH, pH 8, 20 mM sorbitol, and 125 mM KCl) containing either 30% [v/v] of DMSO or 30% [v/v] of ethylene glycol to 3.0 - 5.0 mg Chl/mL. Aliquots (1.0 to 2.0 mL) of chloroplasts or thylakoids were placed either in microcentrifuge tubes or thin walled screw-cap plastic vials and plunged into liquid N₂. Cryopreserved chloroplasts were thawed at room temperature, diluted with import buffer to about 1.0 mg Chl/mL, and repurified on 35% Percoll cushions (Cline, 1986). The percentage of intact chloroplasts was determined from the Chl content of chloroplasts that sedimented through the Percoll relative to the total amount of Chl applied to the cushion. Chloroplasts that sedimented through Percoll were verified to be intact by the ferricyanide reduction assay (Walker et al., 1987). Repurified chloroplasts were washed twice with import buffer before use. Cryopreserved thylakoids were thawed at room temperature, diluted to about 1.0 mg Chl/mL with storage buffer and

recovered by centrifugation at 3200 x g for 8 min. Thylakoid pellets were subsequently washed twice with import buffer, 10 mM MgCl₂ and resuspended in the same buffer to 1.0 mg Chl/mL.

Microscopic Examination of Chloroplast Integrity

Small aliquots of chloroplasts in import buffer were fixed by adding an equal volume of 3% glutaraldehyde in 50 mM potassium phosphate (pH 7.5) and 2 mM MgCl₂. After 45 min on ice, chloroplasts were pelleted and sequentially washed with 50 mM Hepes buffer (pH 8), containing decreasing sorbitol concentrations (0.33 M, 0.16 M, 0.00 M). Chloroplasts were post-fixed with 2% OsO₄ in phosphate buffer (50 mM potassium phosphate, pH 7.5) at room temperature for 2 hours and dehydrated in a graded ethanol series, from 12.5, 25, 40, 60, 75, 85, 95, to 100%, followed by 3 cycles in 100% acetone. Dehydrated chloroplasts were embedded in Spurr's resin (Spurr, 1969) in a graded series of 15%, 30%, 50%, 75%, 90%, and 100% plastic. Thin sections were prepared on an LKB ultramicrotome and were post-stained with uranyl acetate for 20 min followed by lead citrate for 10 min. Sections were examined with a Hitachi HU-11E electron microscope.

Preparation of Radiolabeled Precursors

RNA for pLHCP, pSS, and pPC was prepared by SP6 polymerase transcription of *Eco*RI-linearized plasmids (Cline, 1988) and translated in the presence of [³H] leucine in a wheat germ system (Cline et al., 1989). Translations

were diluted approximately six-fold and adjusted to import buffer containing 30 mM leucine before use.

Assays for Import and Integration of Precursor Proteins

Import assays were carried out *in vitro* essentially as described (Cline, 1986). Each assay contained 200 μL of chloroplast preparation (0.5 mg Chl/mL), 50 μL of adjusted translation product, and 50 μL of 60 mM Mg-ATP in import buffer or 50 μL of import buffer alone (light-driven assays). Assays were initiated with addition of precursor and incubated at 25 °C either in the presence of white light ($\sim 150 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or in darkness. Assays were for 10 min with manual shaking at 5 min intervals. After the reaction, chloroplasts were treated with thermolysin to remove surface-bound precursors, repurified on Percoll cushions, and washed with import buffer containing 5 mM EDTA as described (Cline et al., 1985).

Integration assays with chloroplast lysates were performed basically as described (Cline, 1988). Each assay contained 200 μL of lysates (0.5 mg Chl/mL), 50 μL of 60 mM Mg-ATP in import buffer, 20 mM MgCl_2 or 50 μL of import buffer, 20 mM MgCl_2 alone (light-driven assays), and 50 μL of adjusted translation product. When assays were conducted with reconstituted lysates, reaction mixtures contained 100 μL of thylakoids (1.0 mg Chl/mL) and 100 μL of 2X stromal extract in place of the lysates. Reaction mixtures were incubated at 25°C for 30 min either in darkness or in white light ($\sim 150 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) with manual shaking every 5 min. Thylakoids were

recovered by centrifugation and were further treated with thermolysin to remove non-integrated molecules as described (Cline, 1986).

Analysis of Precursors, Recovered Chloroplasts and Subfractions

Samples of precursors, recovered chloroplasts, and recovered chloroplast subfractions were subjected to SDS-PAGE (Laemmli, 1970). For analysis of the assembly of imported SS into Rubisco, stromal extracts of the recovered chloroplasts were subjected to 6% nondenaturing PAGE (Payan and Cline, 1991). After electrophoresis, gels were prepared for fluorography (Bonner and Laskey, 1974) and placed on X-ray film. Radioactive proteins were extracted from the dried gels (Walter et al., 1981) and quantified as described (Cline, 1986).

Results and Discussion

Preservation of Isolated Intact Chloroplasts

Various compounds have been reported to act as cryoprotectants and to impart protective qualities to biological tissues during low temperature storage (Chen and Li, 1989; Finkle et al., 1985). Among them, DMSO has long been known as one of the most effective cryoprotectants for both plant and animal tissues (Finkle et al., 1985); ethylene glycol, glycerol, and glycine have also been documented as good cryoprotective agents in some cases (Farkas and Malkin, 1979; Finkle et al., 1985). Here, these cryoprotectants at different concentrations were examined for their ability to preserve intact pea chloroplasts. Immediately after isolation, chloroplasts

Table 2-1. Average percentage of intact chloroplasts preserved.

| Protective Substances [v/v] | Percentage |
|-----------------------------|------------|
| Freshly Prepared | 85.0 |
| No Cryoprotectants | 00.0 |
| 10% DMSO | 45.4 |
| 20% DMSO | 67.6 |
| 30% DMSO | 34.5 |
| 10% Ethylene Glycol | 62.7 |
| 20% Ethylene Glycol | 58.3 |
| 30% Ethylene Glycol | 25.3 |
| 10% Glycerol | 20.0 |
| 20% Glycerol | 21.0 |
| 30% Glycerol | 20.0 |

Note: Values in this table were derived from the average of three experiments conducted under the same conditions. Frozen samples were stored overnight in liquid nitrogen (-196 °C).

were resuspended in import buffer containing a cryoprotectant and placed in liquid nitrogen for preservation. Samples were thawed at room temperature and the percentage of intact chloroplasts was determined with a Percoll cushion assay (Methods). The average percentage of intact chloroplasts preserved under different conditions is shown in Table 2-1. Typically, the average percentage of intactness was 85% for freshly prepared chloroplasts. With preserved materials, best results were found to be approximately 65% intact for samples stored in 10% ethylene glycol or in 20% DMSO. For those stored in glycerol, less than 30% intact chloroplasts were preserved at several concentrations, and nearly all chloroplasts were broken when stored in glycine at 0.1 to 0.3 M. Because of the poor preservation of chloroplasts stored in glycerol and glycine, only DMSO- (specifically, 20% [v/v]) and ethylene glycol- (10% [v/v]) preserved chloroplasts were used in subsequent experiments.

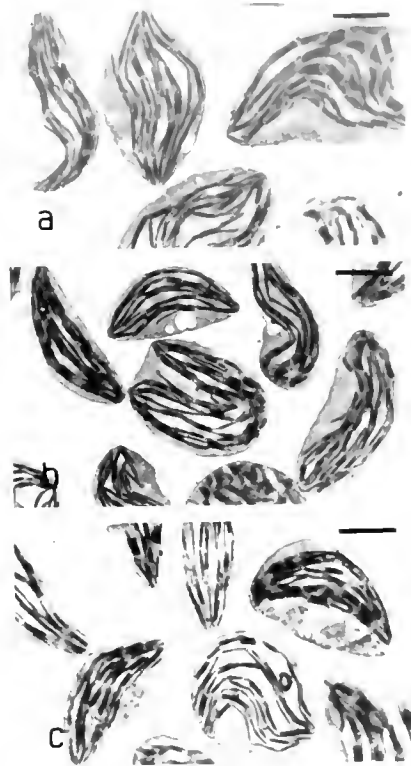


Figure 2-1. Ultrastructural features of fresh and cryopreserved pea chloroplasts. (a) Freshly prepared chloroplasts; (b) chloroplasts preserved in 20% (v/v) DMSO; (c) chloroplasts preserved in 10% (v/v) ethylene glycol. Cryopreserved samples were stored in liquid N₂ for 2 weeks. Bar, 2 μm.

Microscopic Examination of Chloroplast Integrity

Cryopreserved chloroplasts were examined by thin section transmission electron microscopy. Low magnification micrographs gave a good impression of the intactness of chloroplasts (Fig. 2-1). Examination of high magnification photographs revealed no significant differences between freshly prepared chloroplasts and those stored at low temperature in the presence of cryoprotectants (not shown). Outer and inner envelope membranes were present and seemingly unchanged in cryopreserved chloroplasts. Thylakoid structure appeared to be the same in both newly prepared and preserved chloroplasts, with a nearly identical shape and degree of stacking. Percoll cushion repurified samples contained only intact chloroplasts (Fig. 2-1). Examination of unpurified samples revealed that approximately 70% of preserved and 85% of freshly prepared chloroplasts were intact (not shown). These results were fairly consistent with those obtained from Percoll cushion assays (refer to Table 2-1).

Import of pLHCP by Preserved Intact Chloroplasts

Cryopreserved intact chloroplasts were assayed for their ability to import proteins. Both DMSO- and ethylene glycol-preserved intact chloroplasts imported pLHCP into an external protease resistant state (Fig. 2-2). Import could be driven either by light or by exogenous ATP, indicating that preserved chloroplasts had retained both a functional photosynthetic apparatus and a functional adenylate translocator. Almost no pLHCP was imported when assays were carried out in

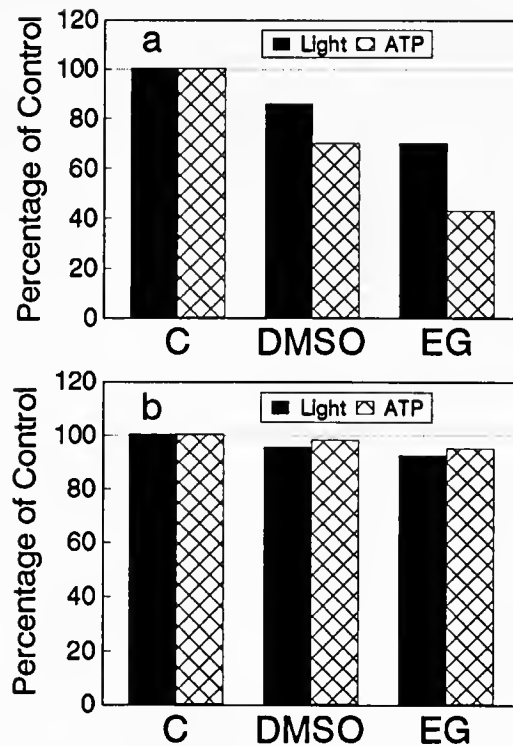


Figure 2-2. (a) Relative import of pLHCP by cryopreserved chloroplasts. Isolated chloroplasts were stored for two days in liquid N_2 in the presence of either 20% [v/v] DMSO or 10% ethylene glycol (EG). Cryopreserved chloroplasts were thawed, repurified, and assayed for their ability to import pLHCP (Methods). Each assay received 2025 molecules of pLHCP per chloroplast. The control (C) was freshly prepared chloroplasts. The absolute number of molecules imported per chloroplast is 323 (light-driven assays) and 227 (ATP-driven assays) for the control. (b) Effects of preincubation with cryoprotectants on import competence. Before use, freshly prepared chloroplasts were incubated either with 20% [v/v] DMSO or 10% ethylene glycol (EG) for 10 min. Incubated chloroplasts were then washed twice with import buffer and assayed for their ability to import pLHCP (Methods). Each assay received 2450 molecules of pLHCP per chloroplast. The control (C) received no cryoprotectants during the 10 min incubation. The absolute number of molecules imported per chloroplast is 398 (light driven assays) and 302 (ATP-driven assays) for the control.

darkness in the absence of ATP (not shown). Furthermore, import ability was virtually abolished by pretreatment of chloroplasts with thermolysin (not shown). These latter observations indicate that import occurred by the physiological mechanism. As import activity is concerned, cryopreserved chloroplasts were up to 85% as active in import assay as freshly prepared chloroplasts. Of the two kinds of cryoprotectants used, DMSO preservation resulted in chloroplasts that were moderately more active. DMSO and ethylene glycol on their own did not significantly affect freshly prepared chloroplasts to import pLHCP.

Correct Assembly of Imported Proteins

Chloroplasts, cryopreserved for 6 months, were used to import several different precursor proteins, including pLHCP, the precursor to the small subunit (pSS) of Rubisco, and the precursor to plastocyanin (pPC). Following import, chloroplasts were recovered and subfractionated into thylakoids and stroma to assess the localization and assembly status of the imported proteins (Fig. 2-3). LHCP was correctly processed to mature size, and properly assembled into the thylakoids as determined by characteristic partial resistance to protease (Cline, 1986). Only a trace amount of LHCP was detected in the stromal fraction. Similarly, mature-sized plastocyanin was recovered with the thylakoid fraction in a protease-protected state. The intermediate-sized plastocyanin precursor (trace amount), which has previously been described (Smeekens et al., 1986), was present in the stromal fraction. Mature-sized SS was recovered with the stromal fraction. Analysis of the stroma by 6%

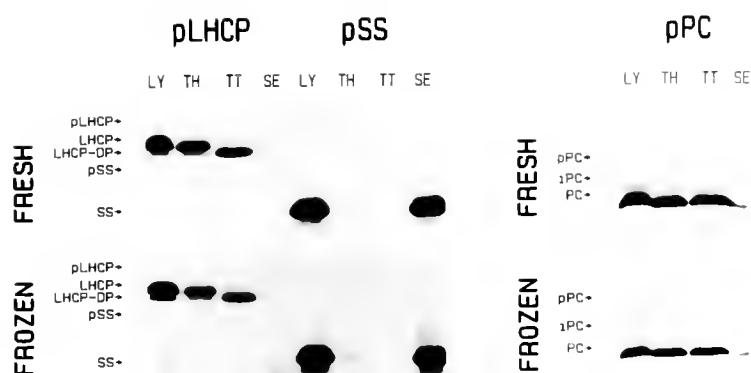


Figure 2-3. Targeting and assembly of proteins imported into cryopreserved chloroplasts. Chloroplasts were cryopreserved with 20% DMSO for 6 months. Chloroplasts were thawed, repurified, and used for import assays (600 μ L) with pLHCP, pSS, and pPC. Following import, chloroplasts were treated with thermolysin to remove surface-bound molecules, repurified on Percoll cushions (Methods), and lysed with 75 μ L of 10 mM Hepes/KOH (pH 8). An aliquot (25 μ L) of each lysate (LY) was removed and the remainder centrifuged at 10,000 \times g for 10 min to separate stroma from thylakoids. Stromal extract (SE) was obtained from the supernatant by further centrifugation (Methods). The pelleted thylakoids were resuspended in 800 μ L of import buffer. Aliquots (400 μ L) of thylakoids were treated with thermolysin at 50 μ g/mL. Thermolysin-treated thylakoids (TT) as well as untreated thylakoids (TH) were then resuspended in 25 μ L of 20 mM EDTA. All samples were analyzed by SDS-PAGE/fluorography. A photograph of the fluorogram is shown.

nondenaturing PAGE demonstrated that SS was assembled into the Rubisco holoenzyme (not shown). These results are virtually identical to those obtained with fresh chloroplasts and demonstrate that the assembly apparatus within chloroplasts has remained functional in the cryopreserved chloroplasts.

Membrane Insertion of (p)LHCP by Chloroplast Lysates

Many of our studies focus on the transport of proteins into and across the thylakoid membranes. These studies utilize an assay in which thylakoid protein transport is reconstituted in chloroplast lysates. Thus, it was important to determine if lysates from preserved chloroplasts were functional in these assays. Cryopreserved intact chloroplasts were lysed and assayed for their ability to integrate (p)LHCP. The results showed that lysates from both DMSO- and ethylene glycol-preserved chloroplasts were capable of integrating (p)LHCP into their thylakoid membranes (Fig. 2-4). Relative integration of (p)LHCP by lysates from preserved chloroplasts was approximately 85% of that by lysates from freshly prepared chloroplasts for ATP-driven assays, and approximately 70% for light-driven assays. As has previously been shown for lysates from fresh chloroplasts (Cline, 1986), virtually no integration occurred when assays were carried out in darkness in the absence of ATP or when stroma was omitted from the assay mixture (not shown). DMSO preservation resulted in lysates that were slightly more active in integration assays than ethylene glycol preservation (Fig. 2-4).

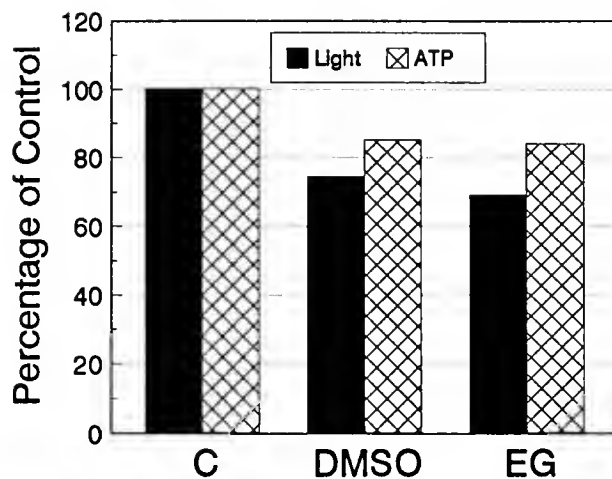


Figure 2-4. Relative integration of (p)LHCP by lysates from cryopreserved chloroplasts. Chloroplasts were cryopreserved overnight in the presence of either 20% [v/v] DMSO or 10% ethylene glycol (EG). Chloroplasts were thawed, repurified, and lysed (Methods). Lysates were assayed for the ability to integrate (p)LHCP into their thylakoid membranes (Methods). Each assay received 5380 molecules of pLHCP per chloroplast equivalent. Control experiments (C) were conducted with lysates prepared from fresh chloroplasts. The absolute number of molecules integrated per chloroplast equivalent is 249 (light-driven assays) and 296 (ATP-driven assays) for the control.

Integration of (p)LHCP by Cryopreserved Thylakoids

Previous studies have identified optimal conditions for the cryopreservation of the photosynthetic competence of isolated thylakoids (Farkas and Malkin, 1979). Similar conditions were examined for their ability to preserve the integration competence of isolated pea thylakoids. Preserved thylakoids were supplemented with fresh stroma and assayed for their ability to integrate (p)LHCP. The results showed that both DMSO- and ethylene glycol-preserved thylakoids were able to integrate (p)LHCP into their membranes (Fig. 2-5). Relative integration by cryopreserved thylakoids was from 95 to 98% of that by freshly prepared thylakoids (ATP-driven assays). For light-driven assays, ethylene glycol-preserved thylakoids were slightly more active than DMSO-preserved thylakoids (Fig. 2-5), suggesting that ethylene glycol may be marginally better than DMSO for stabilizing the photosynthetic apparatus. This result is consistent with previous observation by Farkas and Malkin (Farkas and Malkin, 1979).

Conclusions

In this study, we have described for the first time a simple and very convenient protocol for cryopreservation of isolated intact chloroplasts. By following this protocol, we were able to preserve chloroplasts fully functional for protein import and assembly for six months (Fig. 2-3). We found that DMSO is the superior preserving media for intact chloroplasts and recommend the following protocol. Immediately after isolation of the chloroplasts, the final pellet should be resuspended

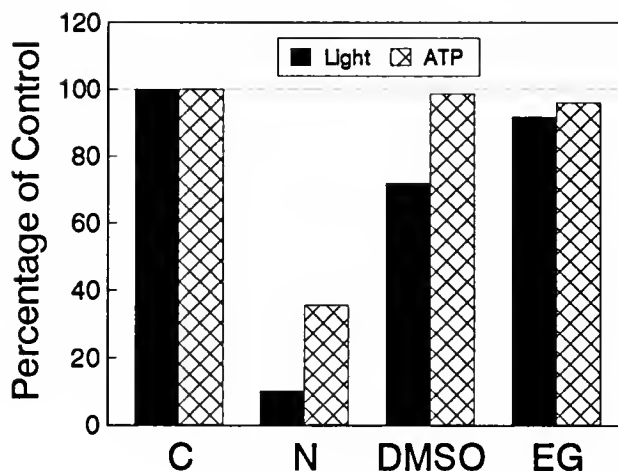


Figure 2-5. Relative integration of (p)LHCP by cryopreserved thylakoids. Isolated thylakoids were cryopreserved for four weeks in storage buffer containing either 30% [v/v] DMSO or 30% ethylene glycol (EG). Preserved thylakoids were thawed, washed, and resuspended in import buffer plus 10 mM MgCl_2 to a Chl concentration of 1.0 mg/mL. Purified thylakoids were furnished with fresh stroma in integration assays. Each assay received 4800 molecules of pLHCP per chloroplast equivalent. The positive control (C) was freshly prepared thylakoids. The negative control (N) was frozen thylakoids with no protective substances. The absolute number of molecules integrated per chloroplast equivalent is 226 (light-driven assays) and 274 (ATP-driven assays) for the positive control.

in import buffer containing 20% DMSO (v/v) at a Chl concentration of 3 to 5 mg/mL. Chloroplasts should then be allowed to equilibrate for about 10 min on ice before freezing. Freezing should be achieved rapidly by submerging small vials in liquid N₂ and thawing be allowed to occur at room temperature. For best results, chloroplasts should be repurified on Percoll cushions before use.

Chloroplasts stored under the above conditions retained their ability to import a variety of different precursor proteins and to correctly localize and assemble them within the chloroplast. These latter processes frequently require transport into or across the thylakoid membrane. The proper localization of plastocyanin and LHCP by cryopreserved plastids, as well as the ability of lysates from these plastids to assemble LHCP demonstrate that the thylakoid transport apparatus has been preserved during low temperature storage. Preserved chloroplasts were also active for *in organello* protein synthesis and produced a similar pattern of labeled polypeptides although in a somewhat reduced amount (not shown). For preservation of isolated thylakoids, a modified method of that reported by Farkas and Malkin (1979) was an effective means of preserving thylakoids for use in protein integration analysis.

We hope that this method will facilitate future studies of chloroplast protein biogenesis. We recommend that investigators test the efficacy of these preservation methods when different precursors or a different source of plastids is to be employed.

CHAPTER 3

STROMAL FACTOR PLAYS AN ESSENTIAL ROLE IN PROTEIN INTEGRATION INTO THYLAKOIDS THAT CANNOT BE REPLACED BY UNFOLDING OR BY HEAT SHOCK PROTEIN HSP70

Introduction

Soluble factors necessary for efficient transport of proteins across or into membranes have been discovered in nearly every translocation system studied in depth. Many soluble factors such as the cytosolic Hsp70, the mitochondrial Hsp60, and the bacterial SecB, DnaK and GroEL are molecular chaperones that function to prevent nonproductive reactions of preproteins such as premature folding (Chirico et al., 1988; Murakami et al., 1988; Bochkareva et al., 1988; Kumamoto, 1989; Phillips and Silhavy, 1990; Koll et al., 1992), aggregation (Lecker et al., 1989), or nonspecific membrane association (Hartl et al., 1990). Certain soluble factors, such as SecB of bacteria and the signal recognition particle (SRP) of mammalian cells, also provide a targeting function. SecB facilitates preprotein targeting to the translocase via its affinity for SecA (Hartl et al., 1990). SRP binds to signal peptide of nascent preproteins (Walter and Blobel, 1982) and targets them to the endoplasmic reticulum (ER) by subsequent binding to the SRP receptor (Gilmore et al., 1982). Some soluble factors, e.g. the bacterial SecA (Schiebel et al., 1991), also participate in the mechanisms of membrane translocation.

The light-harvesting chlorophyll a/b protein (LHCP) is nuclear-encoded and must cross the chloroplast envelope before being integrated into thylakoids. Integration of LHCP into thylakoids has been reconstituted *in vitro* and shown to require thylakoids, ATP, and an as yet unidentified stromal protein factor (Cline, 1986; Fulsom and Cline, 1988). Both the intact precursor (pLHCP) and the mature-sized protein (LHCP) can serve as substrates for the reconstituted reaction (Cline, 1986; Viitanen et al., 1988). Payan and Cline (1991) showed that one function of the stromal factor is to maintain the solubility and integration competence of (p)LHCP (LHCP or pLHCP). The stromal factor accomplishes this by converting (p)LHCP into a large and soluble species, most likely a complex between (p)LHCP and part of the stromal factor. This putative complex has an estimated molecular weight of about 120 kDa. The ability of the stromal factor to convert pLHCP into the 120-kDa complex correlates with the ability of stromal protein to promote pLHCP integration, supporting the idea that complex formation is a step leading to thylakoid integration.

Recently, the precursor form of LHCP has been over-expressed in *E. coli* and the purified pLHCP used *in vitro* to study the importance of soluble factors in protein import into chloroplasts or integration into thylakoids. First, Waegemann et al. (1990) reported that urea-solubilized pLHCP was not competent for import; dialysis of pLHCP with soluble proteins was essential to obtain import competence. Second, Yalovsky et al. (1992) reported that when urea-denatured pLHCP was directly diluted into the integration reaction, it was inserted into thylakoids in the

absence of stromal extract. Further, they reported that stromal extract was required only if urea-denatured pLHCP was dialyzed prior to the integration reaction and that the plastid Hsp70 could replace stromal extract in this reaction, i.e. Hsp70 is the stromal factor.

We have reexamined the soluble factor requirement(s) for pLHCP import and integration using purified *E. coli*-made pLHCP as well as *in vitro*-translated pLHCP. Our studies have shown that whereas unfolded pLHCP is efficiently imported into chloroplasts without cytosolic factors, its integration into thylakoids absolutely requires stromal extract. Hsp70 could not replace stromal extract in the integration reaction and depleting stromal extract of Hsp70 did not impair its ability to support integration, i.e. Hsp70 is not the stromal factor. When considered with the essential nature of stromal extract, the finding that pLHCP is surprisingly stable in aqueous solution indicates that the stromal factor most likely has an active role in the integration reaction.

Materials and Methods

Materials

[³H]-leucine was from Du Pont-New England Nuclear. RNasin and SP6 RNA polymerase were from Promega. Mg-ATP, thermolysin, fast flow DEAE-Sepharose, C-8 linked ATP-agarose, protein A-Sepharose 4B, and Percoll were from Sigma. All other chemicals were reagent grade. Plasmid psAB80XD/4 is the *in vitro* expression plasmid for pLHCP (Cline, 1988). Plasmid pETHpLHCP is the *E. coli* over-

expression plasmid for pLHCP (Cline et al., 1993). Several antibodies were used in this study. The antibody to LHCP has been described (Payan and Cline, 1991). Antibody to the *E. coli* DnaK was kindly provided by John McCarty and Caroline Donnelly (Massachusetts Institute of Technology, Cambridge). Antibody to tomato cytosolic Hsp70 was a gift of Dr. Nover (Neumann et al., 1987).

Preparation of Radiolabeled Precursor, Chloroplasts, Lysates, Thylakoids, and SE

[³H]-pLHCP was prepared either by *in vitro* transcription (Fulsom and Cline, 1988) and translation (Cline, 1986, 1988) or by over-expression in *E. coli* in the presence of [³H]-leucine (Cline et al., 1993). Chloroplasts were isolated from 9- to 10-day-old pea (Laxton's Progress 9) seedlings as described (Cline, 1986; Yuan et al., 1991) and were resuspended in import buffer (50 mM Hepes/KOH, pH 8, 0.33 M sorbitol). Lysates, thylakoids, and stromal extract (SE) were prepared from isolated chloroplasts (Fulsom and Cline, 1988; Yuan et al., 1991). Lysates prepared at 0.5 mg/ml chlorophyll (Chl) were arbitrarily referred to as 1X lysate and the SE resulting from such lysate as 1X SE (~ 3 mg/ml protein).

Purification of Hsp70 from SE and Preparation of Anti-Hsp70 Antibody

Stromal Hsp70 was purified according to Welch and Feramisco (1985) as follows. Stromal protein (100 mg) in buffer A (20 mM Hepes/KOH, pH 8, 20 mM KCl, 5 mM MgCl₂, 1 mM DTT) was loaded at 1 ml/min onto a 10 ml fast flow DEAE-Sepharose column. After the column was washed at 2 ml/min with 30 ml of

buffer A, the bound proteins were eluted at 1 ml/min with a 50 ml, 20 to 500 mM KCl gradient. The fractions containing Hsp70 at ~250 mM KCl were pooled, diluted 5 fold with buffer A, and applied at 10 ml/hour to a 5 ml ATP-agarose column that had been equilibrated with buffer A. The column was sequentially washed with 10 ml buffer A, 20 ml 0.5 M KCl in buffer A, and 20 ml buffer A, and was then eluted with 30 ml buffer A containing 3 mM ATP. The resulting preparation was about 70% Hsp70 and 25% Hsp60 as determined by densitometry of Coomassie blue-stained gels. The identity of Hsp70 was confirmed by specific reaction with antibody against *E. coli* Hsp70/DnaK (Results). Hsp70 was further purified by SDS gel electrophoresis, electroeluted from gel bands, and used for antibody production in rabbits.

Depletion of Hsp70 from SE

Protein A-Sepharose 4B (1 ml aliquot) was washed (Payan and Cline, 1991), suspended in 3 ml of 10 mM Hepes/KOH (pH 8), and then mixed with 3 ml of either 2% bovine serum albumin (BSA), preimmune serum, or anti-Hsp70 serum. The mixture was incubated overnight at 4°C and the supernatant removed by centrifugation at 500 x g for 2 min. After 3 washes (5 ml each) with column buffer (25 mM Hepes/KOH, pH 8, 50 mM KCl, and 10 mM MgCl₂), protein A-Sepharose matrices were transferred into syringe columns with glass fiber filter supports. Stromal extract (2 ml 1X SE) was applied to each column and allowed to pass through after 15 min incubation. The flow-through was reapplied to the column two

more times in a similar fashion and was finally recovered from the column by centrifugation at 500 x g for 3 min.

Assays for Import, Integration, and Soluble Complex Formation

Import assays were conducted for 10 min as described (Cline, 1986; Yuan et al., 1991) except that *E. coli*-made pLHCP was used instead of *in vitro*-translated pLHCP. Assays were started by adding urea-denatured pLHCP to the assay mixture as described in the figure legends. Unless otherwise specified, import reactions (300 μ l) contained chloroplasts equivalent to 0.33 mg/ml Chl, 50 mM Hepes/KOH, pH 8, 0.33 M sorbitol, 10 mM Mg-ATP, 2 mM DTT, 0.2 M or less urea, and approximately 0.2 μ M pLHCP. Integration assays were performed both with *E. coli*-made pLHCP and with *in vitro*-translated pLHCP for 30 min essentially as described (Cline, 1986, 1988). Integration reactions (300 μ l) with *E. coli*-made pLHCP received thylakoids equivalent to 0.33 mg/ml Chl, 9 mg/ml stromal protein, 17 mM Hepes/KOH, pH 8, 55 mM sorbitol, 10 mM Mg-ATP, 1 mM DTT, 0.2 M or less urea, and approximately 0.2 μ M pLHCP. To minimize microcentrifuge tube-adsorbed pLHCP, washed thylakoids recovered from integration assays were transferred to new tubes before analysis. Assays for soluble complex formation were performed according to Payan and Cline (1991).

Sample Analyses

Samples recovered from the above assays were subjected to electrophoresis on 12.5% SDS polyacrylamide gels (Laemmli, 1970) and fluorography (Bonner and Laskey, 1974). About 10% of the chloroplasts or thylakoids recovered from each assay were loaded per gel lane. Quantification of import or integration was accomplished by scintillation counting of radiolabeled proteins extracted from excised gel bands (Cline, 1986; Walter et al., 1981).

Miscellaneous Methods

Chl concentrations were determined according to Arnon (1949). Protein assays were performed by the BCA method (Pierce) for samples without DTT or by the Bradford method (Bradford, 1976) for samples with DTT using BSA as a standard. Immunoprecipitation and immunoblotting were carried out as described (Payan and Cline, 1991).

Results

Stromal Components Are Absolutely Essential for Integration of pLHCP into Thylakoids

Precursor LHCP was produced by over-expression in *E. coli*. The over-expressed pLHCP was sequestered in inclusion bodies. Isolation of inclusion bodies yielded pLHCP that was 90-95% pure as determined by densitometry of Coomassie blue-stained gels. The specific radioactivity of *E. coli*-made pLHCP was 400,000 to 800,000 dpm per μg protein. When purified pLHCP was denatured in 8 M urea and

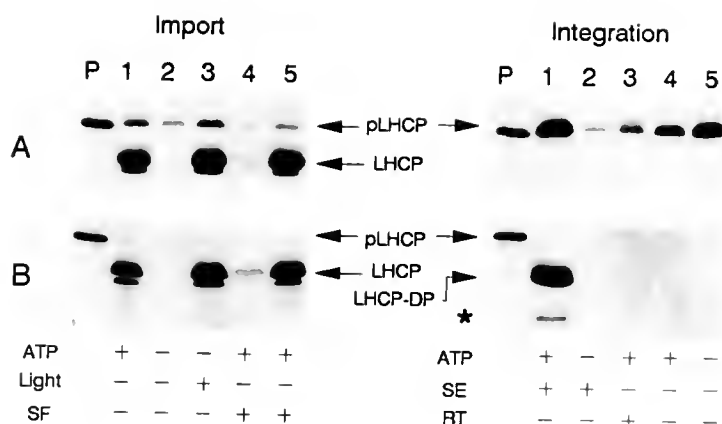


Figure 3-1. Soluble factors are not required for import of purified pLHCP into chloroplasts whereas unfolded pLHCP still requires stromal extract for integration into thylakoids. *E. coli* made-pLHCP was dissolved in 8 M urea, 8 mM DTT at room temperature for 4 hrs and then directly diluted into assays for import into chloroplasts or integration into thylakoids. Import assays were carried out either in dark with 10 mM ATP (lanes 1, 4, and 5), without ATP (lane 2); or in light ($\sim 70 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) with no added ATP (lane 3). Some assays also received soluble factors (SF) i.e. wheat germ protein (lane 4, 1 mg) or rabbit reticulocyte protein (lane 5, 3 mg). Integration assays were carried out in dark with 10 mM ATP (lanes 1, 3, and 4) or without ATP (lanes 2 and 5). Some assays also received 3 mg protein of stromal extract (SE, lanes 1 and 2) or rabbit reticulocyte lysate (RT, lane 3). Recovered chloroplasts and thylakoids were either extracted with 0.1 M NaOH (A) or treated with thermolysin (B). Gels were loaded on an equivalent Chl basis (4.5 $\mu\text{g}/\text{lane}$). Lane P, purified pLHCP. LHCP-DP, a diagnostic protease degradation product of correctly assembled LHCP or pLHCP (Cline, 1988; Andersson et al., 1982; Mullet, 1983). Marker (*) points at a protease degradation product previously characterized as inserted but resulting from incompletely assembled pLHCP (Reed et al., 1990).

then directly diluted into reaction mixtures, it immediately adopted a form competent either for import into chloroplasts or for integration into thylakoids (Fig. 3-1).

Purified pLHCP was able to import into isolated chloroplasts in the absence of any soluble factors as long as energy (ATP or light) was provided (Fig. 3-1B). The *E. coli*-made substrate was imported as efficiently as *in vitro*-translated pLHCP; up to 15% of the added pLHCP was imported during the 10 min incubation. Addition of cytosolic components, i.e. wheat germ extract or reticulocyte lysate, did not stimulate the import of purified pLHCP. On the contrary, elevated quantities of wheat germ extract inhibited import of *E. coli*-made pLHCP (Fig. 3-1, lane 4) as well as *in vitro*-translated pLHCP (unpublished results). Waegemann et al. (1990) previously reported that urea-denatured pLHCP was not competent for import unless it was first dialyzed with cytosolic factors (leaf extract). It is apparent from Waegemann et al. (1990) that cytosolic factors can be helpful to pLHCP import under certain conditions. However, the results in Figure 3-1 demonstrate that they are not essential.

In contrast, integration of purified pLHCP into isolated thylakoids absolutely required the presence of stromal extract in addition to ATP (Fig. 3-1B). Titration of the stromal dependence showed that at least 2.5 mg/ml stromal protein was necessary to obtain appreciable integration (data not shown). In the presence of 9 mg/ml stromal protein, as much as 15% of the added pLHCP was integrated into thylakoids during a 30 minute incubation. Neither wheat germ extract (not shown)

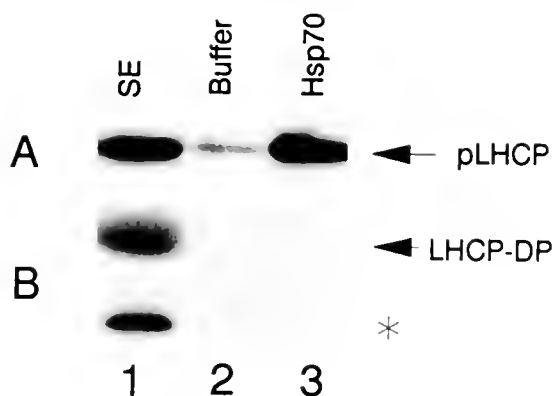


Figure 3-2. Hsp70 is not able to support integration of purified pLHCP into isolated thylakoids. *E. coli*-made pLHCP was solubilized in 8 M urea, 8 mM DTT for 4 hrs at room temperature and then dialyzed to remove the urea before assay for integration. Dialysis was initiated by mixing 10 μ l ($\sim 6 \mu$ g) of urea-solubilized pLHCP with 400 μ l of buffer A containing either 5 mg stromal protein (lane 1), no addition (lane 2), or 100 μ g ATP-agarose affinity-purified stromal Hsp70 (lane 3). Dialysis was conducted at 4°C for 30 min against buffer A on number 3 (molecular weight cut off, 3500 Da) Spectra/por dialysis membrane (Spectrum Medical Industries, Inc., Los Angeles, California). At the end of dialysis, 200 μ l of the dialysis mixture was mixed with ATP, washed thylakoids, and assayed for integration. Recovered thylakoids were treated either by alkali extraction (A) or with thermolysin (B). LHCP-DP and marker (*), see legend to Figure 3-1.

nor rabbit reticulocyte lysate (Fig. 3-1B, lane 3) could replace stromal extract for pLHCP integration.

The above conclusions were made based on protease-resistance of integrated (p)LHCP. It has been shown that membrane-integrated (p)LHCP is largely resistant to protease digestion, yielding a characteristic degradation product (LHCP-DP) upon treatment with proteases (Cline, 1988; Andersson et al., 1982; Mullet, 1983). Using resistance to alkali extraction as a criterion for integration, Yalovsky et al. (1992) concluded that stromal extract was not required for thylakoid integration of denatured/unfolded pLHCP. However, comparison of these two treatments showed that alkali extraction was not sufficiently rigorous to differentiate integrated from surface-bound pLHCP (compare Fig. 3-1, A and B). NaOH-resistant pLHCP was obtained with or without stromal extract, with or without ATP (Fig. 3-1A). Alkali extraction was also not effective in removing pLHCP bound to the surface of intact chloroplasts (Fig. 3-1A). Similar conclusions have recently been reached by two other research groups even with *in vitro*-synthesized pLHCP (Auchincloss et al., 1992; Huang et al., 1992).

Hsp70 Is Not the Stromal Factor Required for pLHCP Integration

Yalovsky et al. (1992) reported that urea-denatured pLHCP lost integration competence upon dialysis unless stromal extract or purified Hsp70 was present during dialysis. We also observed that inclusion of Hsp70 during dialysis of urea-denatured pLHCP led to increased alkali-resistant pLHCP (Fig. 3-2A). However,

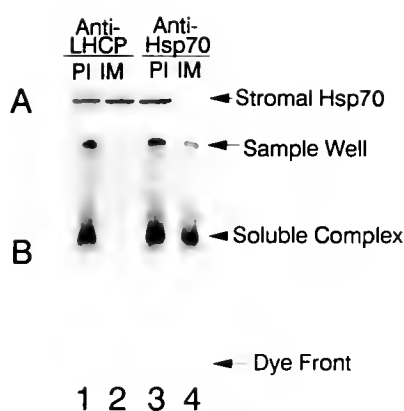


Figure 3-3. Hsp70 is not part of the pLHCP soluble complex. [^3H]-pLHCP (translated *in vitro*) was mixed with stromal extract and incubated for 15 min at 25°C in the presence of 5 mM ATP. The mixture was then subjected to immunoprecipitation analyses either with preimmune (PI) sera (lanes 1 and 3) or with immune (IM) sera (lanes 2 and 4). After removal of the pellet by centrifugation, the supernatant was analyzed for the presence of Hsp70 by immunoblotting (A) or for the presence of the soluble pLHCP complex by native gel electrophoresis (B).

integration of pLHCP as judged by protease-resistance only occurred when stromal extract was present during dialysis (Fig. 3-2B). We suggest that dialysis with Hsp70 increases association of pLHCP with the thylakoids because Hsp70 maintains pLHCP in solution throughout dialysis. In our experiment, most of the buffer-dialyzed pLHCP was adsorbed to the dialysis apparatus.

Payan and Cline (1991) have previously correlated the integration-promoting activity of stromal extract with its ability to convert pLHCP into a 120-kDa soluble complex. They showed that the plastid Hsp60 is not a component of the complex, but could not determine whether the plastid Hsp70 was a component. To assess whether Hsp70 was part of the 120-kDa pLHCP soluble complex, we prepared an antibody to the plastid Hsp70 and used this antibody in immunoprecipitation analyses. Such analyses showed that the pLHCP soluble complex was not removed by treatment with anti-Hsp70 antibody (Fig. 3-3, lane 4). In contrast, all of the complex was removed by treatment with anti-LHCP antibody (Fig. 3-3, lane 2).

The possible involvement of Hsp70 in integration was further assessed by assaying pLHCP integration with Hsp70-depleted stromal extract obtained by immunoaffinity chromatography on an anti-Hsp70 IgG protein A-Sepharose column. Control stromal extract was treated identically either with a preimmune or a BSA mock-treated column. Depletion of Hsp70 was verified by Coomassie blue staining (Fig. 3-4A) and by immunoblotting with anti plastid Hsp70 or anti *E. coli* DnaK antibody (Fig. 3-4B). By using a dilution calibration curve for immunoblotting with the anti plastid Hsp70, we determined that the depletion treatment removed more

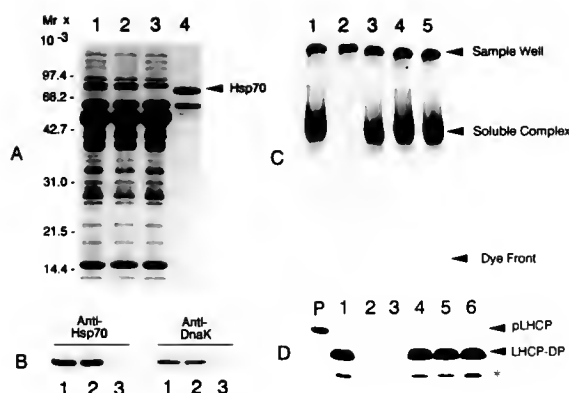


Figure 3-4. Hsp70-depleted stromal extract supports pLHCP integration equally well as does Hsp70-containing stromal extract. Hsp70-depleted stromal extract (SE) was prepared as described in "Materials and Methods". **A.** Coomassie blue staining of stromal protein passed through a BSA mock column (lane 1), passed through a preimmune IgG column (lane 2), passed through an anti-Hsp70 IgG column (lane 3); lane 4, ATP-agarose affinity-purified stromal Hsp70 (shows the enrichment of Hsp70 as well as the presence of Hsp60). **B.** Immunoblotting of stromal protein untreated (lane 1), passed through a BSA mock column (lane 2), passed through an anti-Hsp70 IgG column (lane 3). **C.** Complex formation with SE (lane 1); without SE (lane 2); with SE passed through a BSA mock column (lane 3), passed through a preimmune IgG column (lane 4), passed through an anti-Hsp70 IgG column (lane 5). **D.** Integration with 1X SE (lane 1); without SE (lane 2); with purified stromal Hsp70 (lane 3); with 1.5X SE passed through a BSA mock column (lane 4), passed through a preimmune IgG column (lane 5), passed through an anti-Hsp70 IgG column (lane 6). Lane P, *in vitro*-translated pLHCP. LHCP-DP and marker (*), see legend to Figure 3-1.

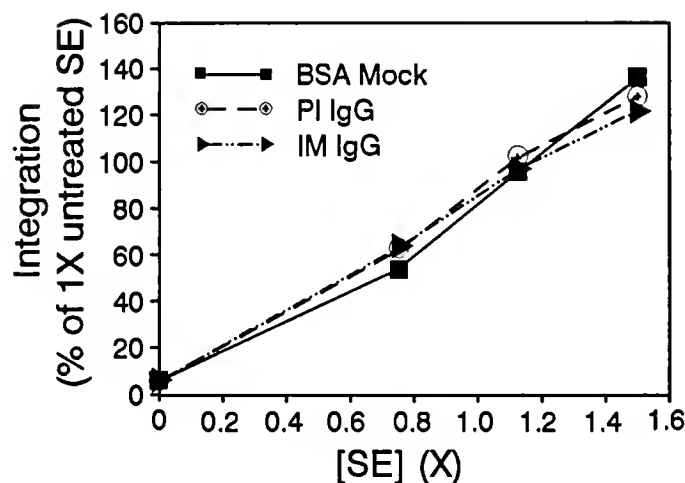


Figure 3-5. Stromal protein concentration-dependent integration of pLHCP is independent of the presence of Hsp70. Quantification of pLHCP integration (see legend to Figure 3-4 for details) with different concentrations (based on protein assays) of stromal extract (SE) that had been depleted of Hsp70 with an anti-Hsp70 IgG column (IM IgG) or treated identically with a preimmune IgG column (PI IgG) or with a BSA mock column (BSA Mock). The protein concentration of 1X stromal extract was 2.45 mg/ml (0.82 mg/ml final concentration in assay). Data are expressed as a percentage of the integration obtained with 1X untreated SE (1000 dpm per μg Chl).

than 95% of the stromal Hsp70. Yet, the Hsp70-depleted stromal extract was as competent as control stromal extract for integrating pLHCP into thylakoids (Fig. 3-4D) or converting pLHCP into the 120-kDa soluble complex (Fig. 3-4C). Marshall et al. (1990) reported that stromal extract contains a second Hsp70. This second Hsp70 is present in very low amounts in stromal extract and, although difficult to visualize by Coomassie staining, can be monitored by immunoblotting with an antibody against tomato cytosolic Hsp70 (Neumann et al., 1987). With a similar dilution calibration curve, we determined that 50-60% of the minor Hsp70 was removed by our depletion treatment (data not shown). In the experiments with depleted stromal extract, the stromal component was the limiting factor for integration (Fig. 3-5). The identical response of pLHCP integration to different amounts of control or Hsp70-depleted stromal extract demonstrates that Hsp70 is not the stromal factor previously described.

pLHCP, Diluted out of Urea, Is Stable as a Substrate for Import and Integration

The notion that unfolded pLHCP is sufficient for integration and that Hsp70 is the stromal factor presupposes that pLHCP rapidly folds into an integration incompetent conformation. We examined this by diluting urea-denatured pLHCP to a low urea concentration (~ 0.2 M) and then assaying aliquots for import and integration at various times after dilution. The amount of import or integration for each time point is plotted in Figure 3-6 as a percentage of the no-preincubation control. The amount of import or integration was about 70% of the no-preincubation

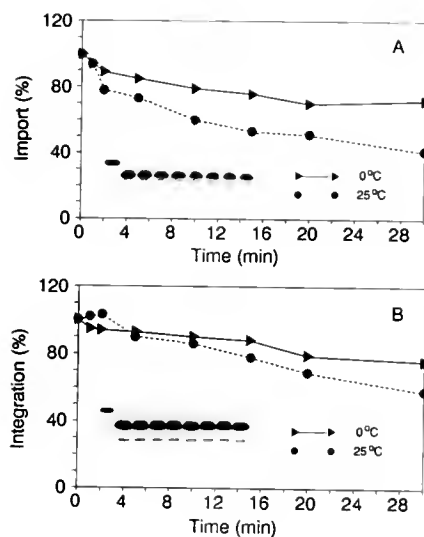


Figure 3-6. Urea-denatured pLHCP remains competent for import and integration during incubation under renaturing conditions. *E. coli*-made pLHCP was denatured in 8 M urea, 8 mM DTT at room temperature for 4 hrs and then diluted to ~ 0.2 M urea into import buffer plus 2 mM DTT. At various times after the dilution, pLHCP was assayed (at a calculated concentration of $\sim 0.2 \mu\text{M}$) either for import into chloroplasts (A) or for integration into thylakoids in chloroplast lysates (B). Preincubation of pLHCP in buffer was performed at 0°C or at 25°C . Assays for both import and integration were carried out in light ($\sim 70 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) in the presence of 10 mM added ATP. The absolute number of pLHCP imported or integrated was, respectively, $\sim 5.5 \times 10^4$ or $\sim 5.7 \times 10^4$ molecules per chloroplast or chloroplast equivalent for the no-preincubation controls. About 14% of the added pLHCP was imported or integrated. Inset in A, fluorogram of import with pLHCP preincubated at 0°C . Inset in B, fluorogram of integration with pLHCP preincubated at 0°C .

control when diluted pLHCP was preincubated for 30 min at 0°C, and around 50% of the no-preincubation control when preincubated for 30 min at 25°C. Thus, although a time- and temperature-dependent decline in import and integration competence occurred, it was not rapid.

We also measured the amount of pLHCP present in each aliquot and found similar declines with time (data not shown). Presumably, pLHCP in 0.2 M urea is prone to adsorption to surfaces such as the plastic tubes and pipet tips used in this experiment. This further supports our earlier assertion (Payan and Cline, 1991) that measurable loss of pLHCP integration competence results from non-productive reactions that lead to insolubility, e.g. aggregation and adsorption, rather than from folding.

Discussion

Our data clearly show that whereas urea-denatured pLHCP is sufficient for import into intact chloroplasts (Fig. 3-1), integration into thylakoids requires a stromal factor that can not be bypassed by unfolding (Fig. 3-1). The apparent integration reported by Yalovsky et al. (1992) in the absence of stromal extract can be entirely explained as unproductively associated pLHCP that was not removed by alkali extraction. Alkali extraction was originally designed to differentiate integral from peripheral membrane proteins in their native forms (Steck and Yu, 1973). Resistance to alkali extraction has traditionally been interpreted as evidence for protein-membrane interaction. However, caution must be taken when such a

criterion is used to assess the behavior of denatured or non-native proteins such as those used in transport studies. In the case of pLHCP integration, the current evidence indicates that the association of pLHCP with thylakoids in the absence of stromal extract is not physiologically relevant.

Our experiments have also demonstrated that Hsp70 is not the stromal factor (Figs. 3-2, 3-4 and 3-5). We cannot rule out a role for Hsp70 in pLHCP integration because of the technical difficulty of removing all Hsp70 proteins from assay mixtures. In the experiment shown in Figures 3-4 and 3-5, Hsp70 proteins (verified by immunoblotting) were associated with the washed thylakoids used in the assays and were present in the wheat germ extract used for preparing the pLHCP. It was necessary to use *in vitro*-translated pLHCP in the depletion experiments because it is not technically feasible to prepare Hsp70-depleted stromal extract sufficiently concentrated for detectable integration of *E. coli*-produced pLHCP, owing to its much lower specific radioactivity. Nevertheless, our results show that plastid Hsp70 is not the stromal factor previously described. First, no integration occurs in the absence of stromal extract even though Hsp70s are present on thylakoids and in the translation mixture (Fig. 3-4). Second, since the stromal factor is limiting in the integration reaction, its removal in total or in part would be reflected in a decrease in integration. As shown in Figure 3-5, there is no difference in the integration-promoting activity between control and Hsp70-depleted stromal extract.

It has been shown that premature folding can prevent membrane transport of a variety of authentic and chimeric preproteins (Liu et al., 1989; Weiss and Bassford,

1990; della-Cioppa and Kishore, 1988; Eilers and Schatz, 1986). However, folding does not appear to be a problem for pLHCP import and integration. The apparent decrease in import and integration competence (Fig. 3-6) can be explained by the time-dependent loss of pLHCP from solution. This is not surprising as a stably folded pLHCP is expected only upon its interaction with the lipid bilayer. A molecular chaperone would help to prevent loss of pLHCP from solution. Indeed, our previous analyses demonstrated a chaperone function for the stromal factor (Payan and Cline, 1991). However, such a role is not essential as demonstrated by the experiment in Figure 3-6, where pLHCP lost only $\sim 50\%$ of its initial competence during an incubation that mimicked the assay conditions for integration. The fact that the stromal factor is absolutely essential (Figs. 3-1 and 3-2) supports the proposal that the stromal factor has an additional function, most probably in targeting pLHCP to the membrane or possibly even in the mechanism by which pLHCP folds into the lipid bilayer.

CHAPTER 4

PLASTOCYANIN AND THE 33K OXYGEN-EVOLVING PROTEIN ARE TRANSPORTED INTO THYLAKOIDS WITH SIMILAR REQUIREMENTS AS PREDICTED FROM PATHWAY SPECIFICITY

Introduction

Plastocyanin (PC) and the 33K subunit of the oxygen-evolving complex (OE33) are two of several thylakoid lumen-located proteins that are made in the cytosol and transported into thylakoids. Recently, competition studies showed that there are two pathways for protein transport into the thylakoid lumen and that PC and OE33 are on the same pathway (Cline et al., 1993). As it has been shown in other systems that transport requirements are intimately related to the mechanisms of the translocation machinery (Neupert et al., 1990; Pugsley, 1993; Gilmore, 1993), our expectation is that PC and OE33 share similar transport requirements.

It is generally agreed that OE33 and PC require ATP for transport (Kirwin et al., 1989; Bauerle and Keegstra, 1991) and that OE33 transport is stimulated by a proton gradient (Mould and Robinson, 1991; Cline et al., 1992a). However, the stromal requirement for transport of PC and OE33 and the involvement of a proton gradient in PC transport are still uncertain. For example, there are conflicting reports on the role of a proton motive force (PMF) in PC transport (Theg et al., 1989; Mould and Robinson, 1991; Cline et al., 1992a).

One problem with previous studies is that the experiments were conducted with intact chloroplasts rather than with isolated thylakoids such that it was more difficult to assess the contributions of different components of the energy requirements and also the requirement for stromal factor(s). The recent development in our laboratory of an *in vitro* assay for PC and OE33 transport with isolated thylakoids has allowed us to investigate the transport properties of these two proteins in more detail. The results show that PC and OE33 are transported into thylakoids with similar requirements; they both require ATP, stromal protein(s) and the trans-thylakoidal proton gradient for maximum transport. These results add support to the conclusions of Henry et al. (1994) and Robinson et al (1994) from analyses of transport of chimeric precursor proteins that transport requirements are intrinsic properties of translocation pathway and not of the translocated protein.

Materials and Methods

Preparation of Precursor Proteins

All reagents, enzymes, and standards were from commercial sources. *In vitro* expression plasmids for pLHCP, pOE33, iOE33 and pPC from pea, and pPC from *Arabidopsis thaliana* have been described (Cline et al., 1993). Radiolabeled precursor proteins were prepared by *in vitro* transcription and subsequent *in vitro* translation in a wheat germ system in the presence of [³H]-leucine (Cline et al., 1993).

Preparation of Chloroplasts, Lysate, Thylakoids, and Stromal Extract

Intact chloroplasts were isolated from 9- to 10-day-old pea (*Pisum sativum* L. cv. Laxton's Progress 9) seedlings (Cline, 1986). Chloroplast lysate, thylakoids and stromal extract (SE) were prepared as described (Yuan et al., 1993). Thylakoids were washed twice with import buffer (50 mM Hepes/KOH, pH 8, 0.33 M sorbitol) plus 10 mM MgCl₂ and finally resuspended in import buffer. When chloroplast lysate was used in assays, chloroplasts were lysed immediately before the assay. Lysate prepared at a chlorophyll (Chl) concentration of 0.5 mg/ml is arbitrarily referred to as 1X lysate and the SE resulting from such lysate as 1X SE (approximately 2.5 mg protein/ml).

Assays for Integration or Transport into Thylakoids

Assays for PC and OE33 transport into isolated thylakoids were conducted for 30 min at 25°C with chloroplast lysate or reconstituted lysate (thylakoids plus SE) equivalent to 50 µg of Chl in a total volume of 150 µl containing 37 mM Hepes/KOH (pH 8), 220 mM sorbitol, 4 mM MgCl₂, and 4 mM Mg-ATP. Assays were terminated by transfer to 0°C, followed by recovery of thylakoids and subsequent protease (thermolysin) treatment to remove surface-bound precursor proteins. Assays for LHCP integration in this study were carried out exactly as for PC and OE33 transport and were different from the original protocol (Cline, 1986) in that the reaction mixture had lower concentrations of sorbitol, ATP, and MgCl₂.

Use of Ionophores and Coupling Factor Inhibitors

Ionophores and the coupling factor (CF₁/CF₀) inhibitor venturicidin were prepared in ethanolic stocks. The CF₁ inhibitor tentoxin was prepared in aqueous stock. Upon addition of ionophores or tentoxin, reaction mixtures were routinely incubated on ice for 10 min prior to assay. Venturicidin was added at 10 μ M and incubated with thylakoids at 25 °C for 10 min before the addition of radiolabeled precursor protein.

Analysis of Precursors, Recovered Chloroplasts and Thylakoids

Samples of precursors, recovered chloroplasts and thylakoids were subjected to SDS-PAGE and fluorography and quantification was accomplished by scintillation counting of radiolabeled proteins extracted from excised gel bands (Cline, 1986).

Miscellaneous

Protein assays were performed by the BCA method (Pierce) using bovine serum albumin as a standard. Chl concentrations were determined according to Arnon (1949).

Results

Transport of PC and OE33 into Isolated Thylakoids

Figure 4-1 shows the results of OE33 and PC transport assayed with chloroplast lysate in light in the presence of added ATP. Transport of PC and OE33

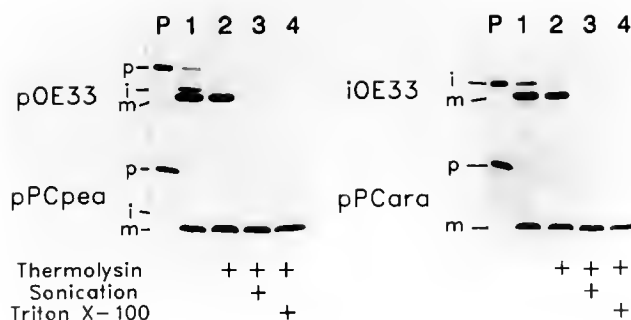


Figure 4-1. Transport of PC and OE33 into thylakoids. Chloroplast lysate (200 μg of Chl) was mixed with radiolabeled protein (pPCara, pPCpea, pOE33, or iOE33) and incubated in the presence of 4 mM Mg-ATP in a total volume of 600 μl of import buffer, 4 mM MgCl_2 in light ($70 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 25°C for 30 min. After the assay, the thylakoids were recovered by centrifugation, washed one time with import buffer, resuspended in 200 μl of import buffer and divided into four equal portions. One portion was mock-treated in import buffer with no addition (lane 1), one portion was treated with 0.2 μg thermolysin per μg Chl for 40 min at 4°C (lane 2), a third portion was sonicated in a bath sonicator during thermolysin treatment (lane 3), and the fourth portion was treated with thermolysin in the presence of 1% Triton X-100 (lane 4). Proteolysis was terminated by addition of EDTA to a final concentration of 50 mM. An equal volume of 2 X SDS sample buffer was added to samples immediately before boiling for 5 min, and about 10% ($\sim 5 \mu\text{g}$ Chl) of each sample was loaded to gels and analyzed by SDS-PAGE and fluorography. Lane P represents 2% of the full-length or the intermediate-sized precursor added to each assay. Positions are indicated for the precursor (p), intermediate (i), and mature (m) forms of the proteins.

into the thylakoid lumen was evidenced by proteolytic processing by the luminal protease to the mature forms of the proteins (lane 1). Several lines of evidence confirm that the appearance of mOE33 and mPC resulted from transport across the thylakoid membrane. The accumulation of mPC and mOE33 was energy dependent (see below), time dependent (increasing for up to 30 min) and temperature dependent, i.e. accumulation of mature forms did not occur at 0°C (data not shown). mPC and mOE33 were found only with the thylakoid pellet upon centrifugation (data not shown). mPC and mOE33 were resistant to added protease (thermolysin) whereas associated precursors or intermediate forms were degraded (lane 2). When the lumen was exposed to protease by sonication or treatment with 1% Triton X-100, mOE33 was degraded (lanes 3 and 4). Unexpectedly, mPC was resistant to degradation under these conditions. A more comprehensive examination with several proteases including trypsin, chymotrypsin, and proteinase K confirmed that mPC is intrinsically resistant to proteolysis (data not shown). A combination of trypsin and chymotrypsin that was reported capable of digesting mPC in an earlier study (Bauerle and Keegstra, 1991) also failed to degrade mPC in our experiments. To verify that mPC was indeed located in the lumen, thylakoids were treated with 0.05% Triton X-100, an amount sufficient to permeabilize thylakoids without solubilizing intrinsic membrane proteins (Ettinger and Theg, 1992; Yuan and Cline, unpublished results). After treatment and centrifugation to pellet the thylakoids, 80% of the mPC was recovered in the supernatant.

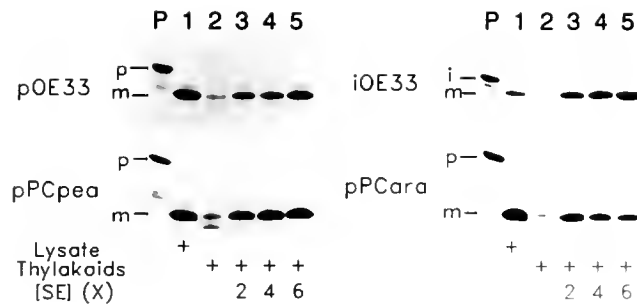


Figure 4-2. Stromal extract stimulates PC and OE33 transport into isolated thylakoids. Radiolabeled pPCara, pPCpea, pOE33, and iOE33 were assayed for transport either with chloroplast lysate (lane 1) or with washed thylakoids supplemented with increasing amount of SE (lanes 2-5) as designated below the panels. About 10% of each sample was loaded to gels. Lane P represents 2% of the full-length or the intermediate-sized precursor added to each assay.

We have observed that up to 28% of pPC_{pea}, 16% of pPC_{Cara}, or 10% of pOE33 added to the reaction mixture was transported into the lumen. However, in most assays the efficiency was about 5% for OE33 and 10% for PC transport. An engineered iOE33 which lacked the stroma-targeting domain and was unable to import into intact chloroplasts (data not shown) was as efficiently transported into thylakoids as pOE33 (Figs. 4-1 and 4-2). This level of transport is significantly less than that for two other luminal proteins OE23 and OE17 (Cline et al., 1992a), but approximately the same as is obtained for integration of LHCP (Cline, 1986; Yuan et al., 1993). We examined transport of PC_{pea} as well as PC_{Cara} in all experiments, but only showed the data for PC_{Cara} in some experiments because of its higher level of translation and overall transport (see below).

Stromal Components Are Necessary for Efficient Transport of PC and OE33 into Thylakoids

It was necessary to include SE in assays for efficient transport of both the full-length precursors (pPC and pOE33) and the intermediate-sized precursor (iOE33) (Fig. 4-2). In general, transport increased with increasing concentrations of SE (Fig. 4-2). With 6X SE (stoichiometry to thylakoids in assay), transport was enhanced approximately 3-fold for OE33 and 2-fold for PC from pea, respectively. The response of PC from *Arabidopsis* was more complex. At relatively low concentrations of SE (e.g. 2X SE), transport of PC_{Cara} was usually stimulated about 2-fold, however, further additions of SE reduced transport (Fig. 4-2, lower right panel).

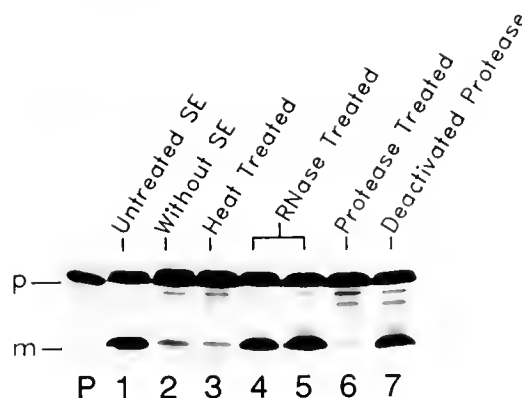


Figure 4-3. SE stimulating activity is resistant to ribonuclease but sensitive to heat and protease treatments. Radiolabeled pPCara was mixed with thylakoids and assayed for transport with SE (lane 1), without SE (lane 2), or with SE treated with heat (lane 3), ribonuclease (lanes 4 and 5) or protease (lanes 6 and 7). Heat treatment of SE (lane 3) was performed for 10 min at 60 °C. Treatment of SE (5 mg/ml) with ribonuclease A at 0.1 mg/ml (lane 4) or at 1 mg/ml (lane 5) was carried out at 25 °C for 20 min. SE became turbid after ribonuclease treatment. Treatment of SE (5 mg/ml) with active (lane 6) or PMSF-inactivated proteinase K (lane 7) at 50 µg/ml was carried out at 4 °C for 30 min. Active proteinase K was deactivated after treatment. Proteinase K was deactivated at 4 °C for 30 min with the addition of freshly prepared PMSF to 10 mM. After treatments, SE was centrifuged on a microcentrifuge for 1 min at 15,000 rpm, 4 °C. Thylakoids recovered from transport assays were washed twice with import buffer without thermolysin treatment. Each lane of the gel received approximately 10% of the recovered samples. Lane P represents 2% of the precursor added to each assay.

To determine the nature of the stromal stimulating activity, SE was pretreated with heat, protease and ribonuclease and then assayed for its ability to stimulate PC and OE33 transport. Treatment of SE with ribonuclease (RNase A) did not affect transport of PC into thylakoids, but treatment with heat or proteinase K abolished its ability to stimulate PC transport (Fig. 4-3). Virtually the same results were obtained with OE33 transport (data not shown). Thus, it appears that the stromal stimulating component is a proteinaceous factor.

ATP Is Essential for PC and OE33 Transport into Thylakoids

Transport of pPC and pOE33 into thylakoids occurred in darkness with exogenously added ATP or in the light without added ATP (Fig. 4-4, lanes 2 and 6). As has been noted before (Cline et al., 1992a), both conditions can result in ATP and a trans-thylakoidal PMF due to the reversible nature of the thylakoidal ATP synthase. Previous work (Cline et al., 1992a) identified conditions for manipulating ATP levels and the PMF in transport assays. Here, we used the same set of conditions for PC and OE33 transport (Fig. 4-4). Parallel assays were also carried out with LHCP as a control to verify the efficacy of the treatments. As can be seen from Figure 4, transport of pPC and pOE33 in light required ATP as evidenced by severe inhibition of transport in the presence of tentoxin (lane 8) or apyrase (lane 9). Apyrase depletes ATP (and GTP) via hydrolysis, whereas tentoxin prevents its formation from photophosphorylation. In experiments designed to determine the nucleotide specificity for PC transport, i.e. using translation products and stromal

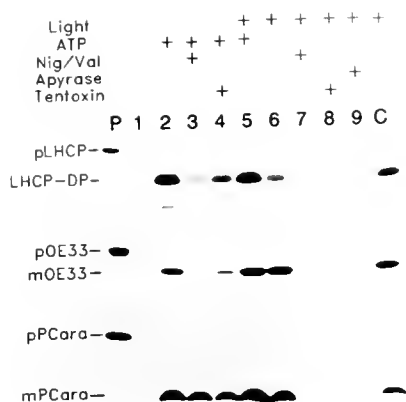


Figure 4-4. Transport of PC and OE33 into thylakoids requires ATP and is stimulated by the trans-thylakoidal PMF. PC and OE33 transport as well as LHCP integration assays were carried out under conditions designed to provide both ATP and a PMF (light, dark + ATP, light + ATP), ATP alone [dark + ATP + nigericin and valinomycin (Nig/Val), dark + ATP + tentoxin], a PMF alone (light + tentoxin, light + apyrase), or neither energetic component (dark, light + Nig/Val). Both integration and transport assays were conducted with chloroplast lysate. Mg-ATP was added to 10 mM final concentration. Apyrase was added to lysate at 2 units per 300 μ l assay and the mixture incubated on ice for 10 min. Tentoxin was to 6 μ M final concentration. Nigericin and valinomycin were added to 0.5 μ M and 1 μ M, respectively, from an ethanolic stock. Control assays received an equal amount of ethanol (lane C). The final ethanol content was 0.33% in assays and was shown to have no effect on the level of transport or integration. About 10% of the samples were loaded to gels for analyses. Lane P represents 2% of the precursor added to the assay reaction.

extract from which all nucleotides had been removed by desalting, we found that ATP was the most effective nucleotide triphosphate but that GTP, CTP, and UTP were also able to support transport (Yuan and Cline, unpublished results). These results indicate that ATP is essential for transport of PC and OE33 into thylakoids. Similar conclusions were also reached by Bauerle and Keegstra (1991) for PC and Kirwin et al. (1989) and Hulford et al. (1994) for OE33 transport.

A PMF Contributes to Transport of PC and OE33 into Thylakoids

The results in Figure 4 also show that the trans-thylakoidal PMF was helpful to transport of PC and OE33 into thylakoids. Inclusion of tentoxin (lane 4) or of a combination of nigericin and valinomycin (lane 3) in assays conducted with ATP in the dark reduced pOE33 transport up to 70% and reduced pPCara (Fig. 4-4) and pPCpea (not shown) transport ~30-40%. The reduction in PC transport was consistently observed whenever the trans-thylakoidal PMF was dissipated with ionophores or the formation of a PMF was prevented with inhibitors of the thylakoidal ATP synthase. This was true when assays were conducted with levels of added ATP from 1 to 10 mM or with SE ranging from 0 to 6X (data not shown). With higher levels of SE, up to 50% inhibition by ionophores was observed for PC transport.

Inhibition of transport by ionophores appears to be a primary effect on the PMF, rather than a secondary effect resulting from the possible ATP depletion by the uncoupled ATPase activity of the thylakoidal ATP synthase. This was shown by

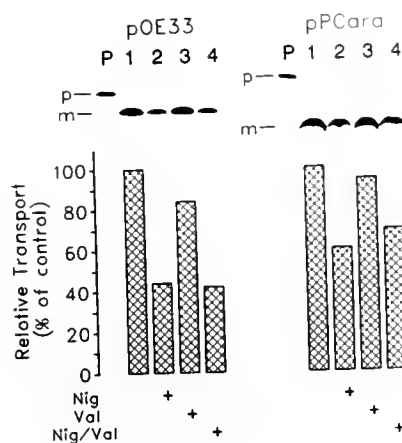


Figure 4-5. ΔpH is the predominant component of PMF in stimulating transport of PC and OE33 into thylakoids. Radiolabeled pPCara or pOE33 was mixed with chloroplast lysate and assayed for transport in darkness at 25°C for 30 min in the presence of 4 mM added ATP. Nigericin at 0.5 μM , valinomycin at 1 μM , or a combination of nigericin (0.5 μM) and valinomycin (1 μM) was added to assays as indicated; transport control reactions received an equivalent amount of ethanol. Approximately 10.6% of pPCara and 5.6% of pOE33 added to assays were transported into thylakoids in the control assays. Lane P represents 2% of the precursor added to the transport reaction.

conducting a time course analysis of transport in the absence or presence of ionophores. If ATP were being depleted, the later stages of the transport reaction would be affected to a much greater extent than the early stages. In fact, during a 40-min transport reaction in the presence of 10 mM Mg-ATP, a combination of 0.5 μ M nigericin and 1.0 μ M valinomycin was equally inhibitory throughout the course of the reaction (data not shown).

Δ pH Is the Active Component of PMF in Stimulating PC and OE33 Transport

To determine the relative contribution of Δ pH and $\Delta\psi$ to transport, individual effects of nigericin and valinomycin were compared (Fig. 4-5). Nigericin acts as an electroneutral H^+/K^+ antiporter and dissipates the proton gradient (Δ pH). Valinomycin functions as an electrogenic K^+ uniporter and collapses the electrochemical gradient ($\Delta\psi$). The results showed that nigericin by itself was as effective in inhibiting PC and OE33 transport as the combination of nigericin and valinomycin, whereas valinomycin alone exerted little if any inhibition, demonstrating that Δ pH is the predominant component of the trans-thylakoidal PMF in stimulating PC and OE33 transport.

PMF Does Not Change the ATP Requirement for PC Transport

Studies of protein export from *Escherichia coli* suggest that PMF alters the response of the translocation machinery to ATP. Specifically, Shiozuka et al. (1990) have shown that in the presence of a PMF, the K_m value of the translocation

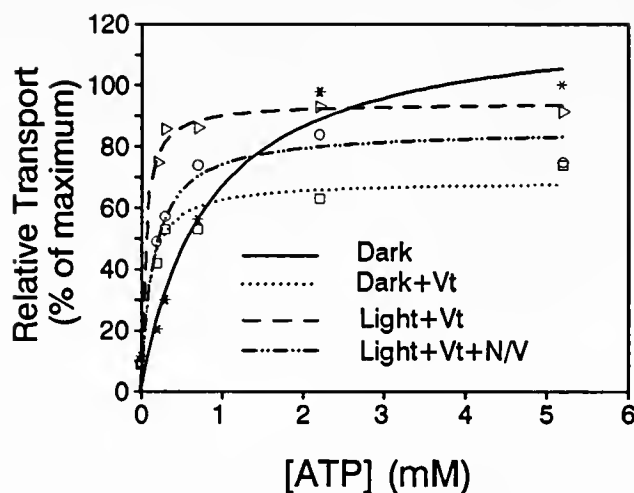


Figure 4-6. ATP concentration curves for PC transport in the presence or absence of a PMF. Radiolabeled pPCara was mixed with chloroplast lysate and assayed for transport in the presence of a PMF (dark, light + venturicidin) or in the absence of a PMF (dark + venturicidin, light + venturicidin + nigericin + valinomycin). ATP was added to assays at 0.0, 0.1, 0.5, 2.0, and 5.0 mM. For the no-ATP control, apyrase at 2 units/assay was included in assays (300 μ l) to deplete the residual amount of ATP (~ 0.2 mM) present in translation mixture and chloroplast lysate. The plotted ATP values were obtained by combining the added ATP with the amount contributed by translation mixture and lysate. Venturicidin (Vt) was added at 10 μ M to prevent formation of a PMF from ATP hydrolysis or to inhibit ATP formation via photophosphorylation. Venturicidin was added to assays from an ethanolic stock; control transport reactions received an equal amount of ethanol. Venturicidin at 10 μ M in the dark completely blocked ATP-derived OE23 transport (data not shown) and was thus effective in preventing Δ pH formation. A mixture of nigericin (N) at 0.5 μ M and valinomycin (V) at 1 μ M was used to dissipate the trans-thylakoidal PMF for light ATP-driven transport reactions. The maximum transport of PCara for this experiment was 940 molecules per chloroplast equivalent. Similar response was observed when this experiment was performed with assay components depleted of ATP (by gel filtration of SE and translation product), but the overall transport activity was too low for quantitative analysis.

reaction for ATP is substantially lower than that in the absence of a PMF. We examined this possibility for thylakoid protein transport by assaying PC transport with varying levels of ATP in the presence or absence of the trans-thylakoidal PMF (Fig. 4-6). For this experiment, the reversible activity of the thylakoidal ATP synthase was inhibited by venturicidin, which blocks the proton pore of CF_0 (Wagner et al., 1989).

The ATP response for PC transport in the presence of a PMF (venturicidin, light) was virtually the same as the response in the absence of a PMF (venturicidin, dark). One difference was that more PC was transported in the presence of a PMF. In both cases transport was saturated by micromolar levels of ATP. If venturicidin was omitted from the dark reactions such that the proton pumping ATPase was active, the maximum amount of PC transported was greater than reactions that contained venturicidin, but much higher levels of ATP were necessary to achieve the maximum transport. These results demonstrate at least three points. First, a PMF *per se* does not influence the quantity of ATP required for the transport reaction. Second, conditions that permit or promote the ATPase function of the thylakoidal ATP synthase lead to higher demands for ATP for the transport reaction, possibly due to depletion of ATP. Finally, the effect of dissipating PMF on transport activity is a primary effect and not due to secondary effect on changing the ATP requirement for transport.

Discussion

Our analyses of PC and OE33 transport into isolated thylakoids show that PC and OE33 transport is ATP-dependent (Figs. 4-4 and 4-6) and is enhanced by stromal protein factor(s) (Figs. 4-2 and 4-3) and the trans-thylakoidal proton gradient (Figs. 4-4, 4-5 and 4-6). The role of stromal factors in OE33 and PC transport has not been entirely clear. Kirwin et al. (1989) reported that wheat OE33 was efficiently transported into pea thylakoids in the absence of SE. Mould et al. (1991) subsequently reported that efficient transport of OE33 into thylakoids required the presence of SE, but suggested that the intermediate-sized OE33 was the substrate for thylakoid transport and that it was the processing protease activity of SE that stimulated OE33 transport. It can be seen from Figure 2 that transport of pea iOE33 as well as pOE33 into pea thylakoids was greatly stimulated in the presence of SE. The fact that iOE33 also depends on SE for efficient transport demonstrates that stromal protein factor(s) perform some function other than processing. This agrees with recently published studies of Hulford et al. (1994) that SE is required for transport of wheat iOE33.

Bauerle and Keegstra (1991) reported that SE inhibited PC transport into isolated thylakoids. They provided evidence that the full-length PCara precursor was the active substrate for transport into thylakoids and that the intermediate-sized forms of PCara were not active in thylakoid transport. They concluded that the processing activity of SE was responsible for the inhibition of transport in the presence of SE because the stromal processing enzyme converted some of the PC

precursor into intermediate-sized forms of the protein. Our results show that SE stimulates transport of *Arabidopsis* PC as well as pea PC. However, there did appear to be a stromal component that was inhibitory to *Arabidopsis* PC transport because elevated levels of SE resulted in less transport than lower levels of SE (Fig. 4-2, compare lane 2 with lanes 4 and 5). Other studies from our laboratory support the notion that pPCara is the substrate for transport across pea thylakoid membrane. When a time course for pPCara chloroplast import and assembly was examined using a rapid stopping technique, the full-length precursor was the only species within chloroplasts that exhibited the kinetics expected for a pathway intermediate (Cline et al., 1992b). Furthermore, it was predominantly pPCara that accumulated in chloroplasts when thylakoid transport was competed by saturating concentrations of iOE33. These were not observed for pPCpea, where intermediate-sized species displayed pathway kinetics (unpublished results of C. Li and K. Cline) and accumulated during competition studies (Cline et al., 1993).

There has also been some confusion regarding the role of the trans-thylakoidal proton gradient in PC and OE33 transport. Theg et al. (1989) and Cline et al. (1992a) failed to detect effects of ionophores on the localization of PC during import into intact chloroplasts, whereas Mould and Robinson (1991) reported partial inhibition of PC localization by ionophores. Earlier, Mould and Robinson (1991) reported that a proton gradient is required for transport of OE33 into thylakoids. Yet, recent studies from the same laboratory (Nielsen et al., 1994) failed to detect stimulation of OE33 transport by the Δ pH. Previously, Cline et al. (1992a) and

Henry et al.(1994) reported that transport of OE33 into thylakoids is partially inhibited by ionophores during import into intact chloroplasts. Here, we show that ionophores cause severe reduction (60-70%) of OE33 transport and a small but reproducible reduction (30-40%) of PC transport with isolated thylakoids (Figs. 4 and 5). We think it likely that due to the fact that ΔpH is stimulatory in these *in vitro* assays, the effect of ΔpH may not be detected in certain experiments due to specific methods of conducting the assays. For example, in the studies of Cline et al. (1992a) and Henry et al. (1994), rapid stopping methods had to be used to avoid subsequent transport of iOE33 during work-up procedures. Certainly, the relatively small contribution of a ΔpH to PC transport together with the fact that it is more difficult to assess the role of the PMF on thylakoid transport with intact chloroplasts may explain previous discrepancies regarding the involvement of a PMF in PC transport.

Even though ΔpH is only stimulatory to PC and OE33 transport *in vitro*, it may play an essential role in their transport *in vivo*. In bacteria such as *E. coli*, signal peptide-bearing proteins are exported by a transport system that depends on SecA and SecY/E proteins and is powered by the combined action of ATP hydrolysis and a PMF (Pugsley, 1993). SecA and ATP are essential and necessary for the initial insertion of the precursor across the bilayer (Schiebel et al., 1991). Under certain *in vitro* conditions, SecA and ATP can complete the transport in the absence of a PMF. Thus, it is no wonder that a wide range of stimulatory effects of PMF has been reported for bacterial protein export *in vitro*, whereas PMF appears to be

essential *in vivo* (Mizushima and Tokuda, 1990).

Because of the endosymbiotic origins of chloroplasts, protein transport into thylakoids has frequently been assumed to occur by mechanisms similar to those for protein export in bacteria (Smeekens et al., 1990). In this regard, we notice that transport of PC and OE33 exhibits similar energy requirements to those of the SecA-dependent protein translocation system in *E. coli*. Recent studies have also shown that PC and OE33 transport is sensitive to azide (Cline et al., 1993; Henry et al., 1994; Knott and Robinson, 1994; Yuan et al., In preparation), a characteristic inhibitor of the SecA ATPase activity (Oliver et al., 1990). SecA homologous genes have been found in the plastid genomes of two algal species (Scaramuzzi et al., 1992; Valentin, 1992). Although secA homologues have not been found in plastid genomes of land plants, we have recently detected a polypeptide of ~ 110 -KDa in pea chloroplasts that is immunoreactive with an antibody raised against a conserved region of an algal chloroplast SecA (Yuan et al., in preparation). The results here, demonstrating that OE33 and PC share similar requirements for transport across the thylakoid membrane, are consistent with competition studies that indicate that OE33 and PC share at least one component of the translocation machinery (Cline et al., 1993). It is possible, but remains to be directly demonstrated that OE33 and PC use a common SecA-homologous apparatus for thylakoid transport.

CHAPTER 5

ONE OF THREE PATHWAYS FOR PROTEIN TRANSPORT INTO PEA THYLAKOIDS USES A *SECA*-DEPENDENT TRANSLOCATION MECHANISM

Introduction

Because of the endosymbiotic origin of chloroplasts from an ancestor cyanobacterium, protein transport into plant thylakoids has been speculated to resemble protein export across the cytoplasmic membrane in prokaryotes (Hartl and Neupert, 1990; Smeekens et al., 1990). Indeed, evidence suggesting a thylakoid system homologous to bacterial protein transport has been accumulating. First, the lumen-targeting domains of thylakoid luminal precursor proteins are similar to prokaryotic signal sequences (Von Heijine et al., 1989) and have been shown to function as such in *E. coli* (Seidler and Michel, 1990; Anderson and Gray, 1991). Second, the cleavage specificity of the luminal peptidase that removes thylakoid targeting signals is identical to that of *E. coli* signal peptidase (Halpin et al., 1989). Third, the energy and soluble factor requirements especially for PC and OE33 transport are very similar to those of the SecA-dependent translocation system (see the results in chapter 6 of this dissertation).

Azide has been shown to be a specific inhibitor of bacterial protein export due to its interference with the translocation ATPase of SecA (Oliver et al., 1990). We

first reported in our discussion that sodium azide specifically inhibits transport of PC and OE33 but not of OE23 and OE17 or integration of LHCP into thylakoids (Cline et al., 1993). Later, Knott and Robinson (1994) confirmed that azide reversibly blocked PC and OE33 transport but was without effect on OE23 transport. Henry et al. (1994) demonstrated that azide sensitivity is a pathway specific feature by analyzing the transport of chimeric proteins. These results suggest that the PC and OE33 pathway uses a translocation mechanism similar to that of the bacterial SecA-dependent translocation system. This notion is further supported by the discovery of secA and secY gene homologues in the plastid genome of red algae (Sccramuzi et al., 1992a, 1992b; Valetine, 1993). However, previous efforts with antibodies to *E. coli* SecA and by use of *E. coli* SecA to support transport were unsuccessful to demonstrate a SecA involvement in the transport of proteins into thylakoids.

As an alternative approach to the identification of a SecA homologue in plant chloroplasts, we overexpressed a highly conserved segment of an algal SecA and produced antibody to this conserved peptide. This antibody reacts with SecA from *E. coli* and also with a pea chloroplast protein of ~ 110 kDa on SDS-PAGE gels, slightly larger than the *E. coli* SecA (102 kDa). The chloroplast SecA (CPSecA) is present in low abundance in chloroplasts and mainly found in the soluble fraction. Using immunoblotting as an assay, we have purified CPSecA from stromal extract by a combination of conventional purification methods. Purified CPSecA was able to support transport of PC and OE33 in an azide-sensitive fashion. In contrast, purified CPSecA had no ability to support integration of LHCP or to stimulate

transport of OE23 and OE17 into thylakoids. Taken together, our studies have defined one of the pathways for protein transport into thylakoids to be a chloroplast SecA-dependent translocation pathway. Our studies also provide strong evidence for conservative sorting of intra-chloroplast proteins. In addition, our strategy to the identification of CPSecA should be of general use in the search of homologous proteins for researchers from other field.

Materials and Methods

Chemicals and Expression Plasmids

Sodium azide, phenylmethyl sulfonylfluoride (PMSF), DEAE-sepharose (fast flow), Sephacryl S-300 HR were from Sigma. Ammonium sulfate was from Fisher. Hydroxylapatite was from BioRad. Mono Q HR 5/5 and Superose 6 HR 10/30 were from Pharmacia. Hydropore-HIC hydrophobic interaction column was from Rainin. Other reagents, enzymes, and standards were all from commercial sources. Plasmid pMAQ805, which contains the complete coding sequence of the Pavlova lutherii secA homologue, was kindly provided by Dr. Harold W. Stokes of Macquarie University, Australia (Scaramuzzi et al., 1992). The *in vitro* expression plasmids for wheat pOE33 and iOE33 were provided by Dr. Colin Robinson (Hulford et al., 1994). The *in vitro* expression plasmids from pea and *Arabidopsis* were described earlier (Cline et al., 1993; Yuan and Cline, 1994).

Cloning of A Conserved Segment of SecA into An *E. coli* Overexpression Plasmid

SecA protein sequences from *P. lutherii* (Pvlepseca), *Antithamnion* spp (Asplas), *E. coli* (Ecoseca) and *Bacillus subtilis* (Bacsubseca) were retrieved from the GenBank database. The sequences were aligned using the GCG program PILEUP (Devereux et al, 1984). A highly conserved region was identified by visual inspection. A clone of the *P. lutherii* chloroplast secA was obtained (Scaramuzzi et al., 1992). The DNA fragment containing the conserved region from bp 1043 to 1458 was amplified by polymerase chain reaction (PCR) with a 5' primer 5'-GCTCCACCATA-TGAAAATCGCCGAGATGAAGACAGG-3' containing an in-frame *NdeI* site and a 3' primer 5'-GGAATGTTTCAAGCTTTCGGGAGATTATTAGTGG-3' containing a *HindIII* site to allow in-frame fusion of the 3' His-6 tag in pET24b. After digestion with *NdeI* and *HindIII*, the PCR product was ligated with *NdeI* and *HindIII* digested pET24b vector by standard procedures (Crowe et al., 1991). Three positive clones designated pJYsecA were obtained and confirmed to be the same with no error in their DNA sequence. For expression of JYsecA peptide (~16-kDa), pJYsecA was introduced into *E. coli* BL21(ΔDE3).

Expression of JYsecA in *E. coli* and preparation of anti-JYsecA antibody

Cells harboring pJYsecA were grown in LB medium to an OD at 600 nm of ~1.0. Isopropyl thio-β-D-galactoside (IPTG) was added to 2 mM and the cells were allowed to grow for another 4 hrs at 37°C. Cells were harvested by centrifugation and checked for expression of JYsecA. After induction, JYsecA accounted for about

5% of the *E. coli* protein and was found to be in inclusion bodies. Inclusion bodies were isolated as previously described (Cline et al., 1993). Inclusion bodies were solubilized in 8 M urea and JYsecA purified on a nickel column equilibrated with 6 M urea according to instructions provided by the manufacturer. For antibody preparation, JYsecA was further purified by SDS-PAGE and electroeluted from excised gel band. Antibody to SDS-denatured JYsecA was prepared by Cocalico Biological Chemical Cooperation (PA).

Purification of CPSecA

CPSecA was purified from SE by a combination of ammonium sulfate precipitation, DEAE-sepharose ion exchange, hydroxylapatite selective adsorption, Sephacryl S-300 gel filtration, Mono Q anion exchange and Hydropore hydrophobic interaction chromatographies. Fractions were monitored for CPSecA with antibody to JYsecA. For ammonium sulfate precipitation, 30 grams of ammonium sulfate solid was added to 260 mls of 2X SE (about 1.3 grams of protein) for the first cut (20% saturation) to precipitate membrane vesicles and denatured proteins. After removing the precipitated material by centrifugation, another 33 grams of ammonium sulfate solid was added to the supernatant to reach a 40% saturation of ammonium sulfate that is necessary to precipitate CPSecA protein. The precipitated proteins were recovered by centrifugation and dissolved in Buffer A (25 mM Hepes/KOH, pH 8, 50 mM KCl, 5 mM MgCl₂) plus 1 mM PMSF. The sample (120 mls) was then applied to a 90 ml DEAE-sepharose column equilibrated with Buffer A. The column

was washed with 200 mls of Buffer A, followed by elution with 500 mls of a salt gradient (50-350 mM KCl). Fractions (5 ml) were immunoblotted with anti-JYsecA and those with CPsecA were pooled for hydroxylapatite chromatography. An equal volume of Buffer A was mixed with the pooled sample (25 mls) and then applied to a 45 ml column of hydroxylapatite equilibrated with 10 mM potassium phosphate (pH 7). After wash with 100 mls of 10 mM potassium phosphate (pH 7) and 100 mls of 1 M KCl (unbuffered), the column was eluted with 300 mls of a gradient of potassium phosphate (10-300 mM). Fractions (3 ml) were immunoblotted for the presence of CPsecA and those with CPsecA were pooled and concentrated to 1 ml by centrifugation through Centriprep-10 (Amicon, Inc.). The concentrated sample was then applied to a 80 ml Sephacryl S-300 gel filtration column equilibrated with gel filtration buffer (20 mM Hepes/KOH, pH 8, 65 mM KCl, 1 mM DTT, 1% ethylene glycol). Fractions (1.5 ml) were blotted for the presence of CPsecA and those with CPsecA were pooled for anion exchange on a 1 ml Mono Q column equilibrated with starting buffer (20 mM Hepes/KOH, pH 8, 50 mM KCl). After wash with 10 mls of starting buffer, the column was eluted with 30 mls of a salt gradient (50-300 mM KCl). Fractions (0.5 ml) were stained for the presence of CPsecA and those with CPsecA were pooled and adjusted to PAS buffer (50 mM potassium phosphate, pH 7, 1.5 M ammonium sulfate) for hydrophobic interaction on a 7.85 ml polyethylene glycol matrix Rainin's Hydropore-HIC column equilibrated with PAS. After wash with 20 mls of PAS, the column was eluted with 50 mls of a descending salt gradient (1.5-0 M ammonium sulfate).

Use of Sodium Azide

Sodium azide was prepared in aqueous stock at 0.6 M in import buffer. Chloroplasts or thylakoids were incubated with sodium azide for 10 min at 25°C in light ($70 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) prior to the addition of precursor to assay mixture. Sodium azide was used at the concentrations specified in the text and figure legends.

Miscellaneous Methods

Chloroplasts were isolated from 9- to 10-day-old pea (Laxton's Progress 9) seedlings as described (Cline, 1986). Chl concentrations were determined according to Arnon (1949). Chloroplast lysate, SE and thylakoids were prepared as described (Cline et al., 1992; Yuan et al., 1991). Assays for import into chloroplasts or for integration/transport into thylakoids were carried out as previously described (Cline et al., 1993; Yuan and Cline, 1994). Sample analysis was carried out according to Cline (1986). Immunoblotting was carried out as described (Payan and Cline, 1991). Protein assays were performed by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Results and Discussion

Effects of Azide on Protein Transport into Thylakoids

Azide is a specific inhibitor of protein export from bacteria (Oliver et al., 1990). To examine whether protein transport into thylakoids is analogous to protein export from bacteria, we investigated the effects of azide on transport or integration

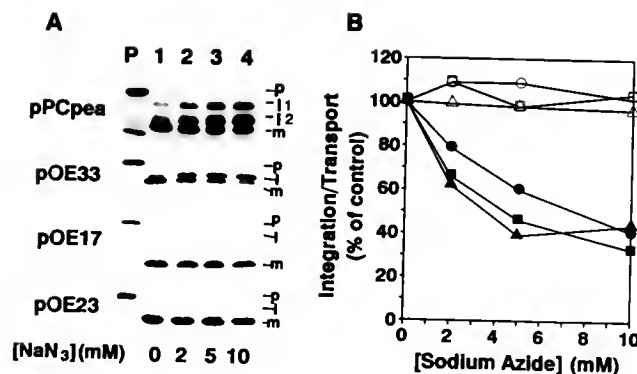


Figure 5-1. Effect of azide on protein transport into thylakoids assessed with intact chloroplasts during import (A) or with isolated thylakoids during transport (B). Radiolabeled precursors (p) of PC, OE33, OE23 and OE17 from pea were assayed for import and subsequent localization with intact chloroplasts in the absence or presence of increasing concentrations of sodium azide. Accumulation of the mature forms (m) of the proteins indicates that the imported proteins have been transported into thylakoids. Accumulation of intermediate species (i) indicates that thylakoid transport of the imported proteins was inhibited. Import assays were terminated with HgCl₂ (Reed et al., 1990). Transport of PC (■), OE33 (▲), OE23 (□) and OE17 (Δ) from pea and PC (●) from *A. thaliana* and integration of pLHCP (○) from pea into thylakoids were carried out with chloroplast lysates in the absence or presence of increasing concentrations of sodium azide.

of proteins into thylakoids. Figure 5-1A shows the results of an import experiment carried out in the presence of increasing concentrations of sodium azide. Import of proteins into chloroplasts was demonstrated by processing of the precursors to their mature or stromal intermediate forms and by co-purification with intact chloroplasts. Import of all four luminal proteins (PC, OE33, OE23, and OE17) into chloroplasts was not significantly affected by sodium azide at concentrations used in the experiment. However, transport of the imported PC and OE33 into thylakoids was severely inhibited in the presence of sodium azide, leading to the accumulation of PC and OE33 stromal intermediates. In contrast, the localization of OE23 and OE17 was unaffected.

The effect of azide on transport of PC, OE33, OE23, and OE17 into the thylakoid lumen and on integration of LHCP into the thylakoid membrane was also assessed in a reconstituted reaction with isolated thylakoids (Fig. 5-1B). The results showed that while transport of PC and OE33 was inhibited in the presence of sodium azide, integration of LHCP and transport of OE23 and OE17 were not affected by sodium azide. The fact that azide does not interfere with LHCP integration or with OE23 and OE17 transport is consistent with competition results that they utilize different pathways for transport into thylakoids. In bacteria, azide has been shown to target exclusively to the SecA protein and its inhibitory effect on protein transport is attributed to its interference with the SecA ATPase activity (Oliver et al., 1990). The fact that sodium azide specifically inhibits PC and OE33 transport suggests that

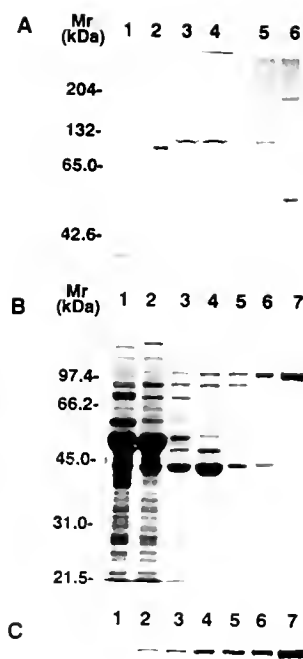


Figure 5-2. Detection and purification of a SecA homologue from pea chloroplasts. (A) Immunoblotting of *E. coli* and chloroplast protein with an antibody to a conserved peptide deduced from an algal *secA* gene "Materials and Methods". Lane 1, total *E. coli* protein from a wild type strain; lane 2, soluble extract of *E. coli* cells harboring the SecA-overexpressing plasmid pT7-secA (Schmidt and Oliver, 1989); lane 3, total chloroplast protein; lane 4, stromal protein; lane 5, thylakoid protein; lane 6, total envelope protein. Total envelope membranes were isolated according to Cline (1985). (B) Protein profile of the Coomassie blue-stained gel of the CPSecA-containing fraction from each purification step. Lane 1, total stromal protein (12.5 μ g); lane 2, ammonium sulfate precipitation (11.5 μ g); lane 3, DEAE-Sepharose ion exchange (3.5 μ g); lane 4, hydroxylapatite chromatography (5 μ g); lane 5, Sephacryl S-300 gel filtration (1.75 μ g); lane 6, Mono-Q anion exchange (1 μ g); lane 7, Hydropore hydrophobic interaction (2 μ g). (C) An immunoblot of the samples shown in the Coomassie blue-stained gel.

they are transported by a similar mechanism to that of the bacterial SecA-dependent translocation system.

Identification and Purification of a SecA Homologue from Pea Chloroplasts

A crucial step to demonstrate the involvement of a SecA type of system for the transport of proteins into thylakoids is to identify a SecA homologue and to demonstrate its involvement in protein transport into thylakoids. As antibody against the entire SecA protein from *E. coli* failed to react with anything from plant chloroplasts (Knott and Robinson, 1994), our strategy was to make an antibody to a conserved segment of SecA and use that antibody to probe chloroplast proteins. For this, protein sequences from all known SecAs (2 from bacteria and 2 from algal chloroplasts) were aligned and compared for similarities. Eight conserved regions were identified and 4 of them are clustered in a relatively small segment (128 amino acid residues) of the SecA protein. This segment, from residue 95 to 222 of the *P. lutherii* chloroplast secA gene, contains sites identified in *E. coli* for ATP binding and azide sensitivity suppression (Oliver et al., 1990), and a site in *B. subtilis* SecA necessary for functional complementation of *E. coli* SecA mutants (Klose et al., 1993).

Antibody raised against this peptide reacted with SecA from *E. coli* (Fig. 5-2A, lanes 1 and 2) and also with a protein of ~110 kDa (Fig. 5-2A, lanes 3-6) from pea chloroplasts. The chloroplast SecA (CPSecA) was found mainly in the stromal fraction of the chloroplast, but was present at low levels in the thylakoid and

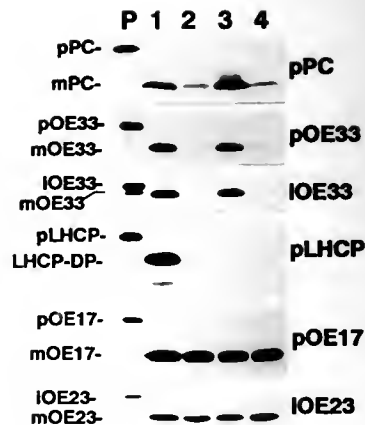


Figure 5-3. Reconstitution of protein transport with purified CPSecA and isolated thylakoids. Radiolabeled full-length or intermediate-sized precursor proteins were mixed with thylakoids and assayed for transport or integration with total stromal protein (315 $\mu\text{g}/\text{assay}$, lane 1), in the absence of soluble protein (lane 2), with purified CPSecA (1.2 $\mu\text{g}/\text{assay}$, lane 3), or with a stromal fraction from gel filtration that lacked CPSecA (26 $\mu\text{g}/\text{assay}$, lane 4). Purified CPSecA was prepared for assay by buffer exchange on a Superose 6 gel filtration column. Assays were conducted as described (Yuan and Cline, 1994) except that each received 4 mM ATP and 4 mM GTP. All assays were 75 μl and contained the same buffer composition. Radiolabeled proteins used in this experiment were pPC from *A. thaliana*, pOE33 and iOE33 from wheat (Hulford et al., 1994), pLHCP, pOE17 and iOE23 from pea.

envelope membrane fractions. By using immunoblotting as an assay, CPSecA was purified from SE by a combination of conventional chromatographies (see 'Materials and Methods' for purification details). Like the bacterial SecA, CPSecA exists as a dimer in solution, migrating on gel filtration columns with a size of 200-25 kDa. The purity of CPSecA from each step is shown in Figure 5-2B. Immunoblotting of the pooled sample from each step is shown in Figure 5-2C. From a dilution series it is estimated by immunoblotting that about 0.4% of the stromal protein is CPSecA. Thus, CPSecA was enriched approximately 250 fold.

Purified CPSecA Is Able to Support Transport of PC and OE33 into Thylakoids

Purified CPSecA was tested for its ability to stimulate transport of PC and OE33 into thylakoids. A gel filtration fraction that has much more stromal protein but contains no CPSecA was used as a control to demonstrate specificity of CPSecA in supporting transport. The results showed that purified CPSecA was able to replace SE in supporting transport of PC and OE33 into isolated thylakoids (Fig. 5-3). CPSecA, on the other hand, showed no activity to support LHCP integration into the thylakoid membrane or to stimulate OE23 and OE17 transport across the membrane into the lumen. Transport of PC and OE33 into thylakoids increases with increasing concentrations of CPSecA protein and approaches saturation at about 150 nM (1.25 µg/75 µl assay) of CPSecA monomer (Fig. 5-4A). Sodium azide inhibits CPSecA-dependent transport of PC and OE33, demonstrating that CPSecA is the azide sensitive component (Fig. 5-4B).

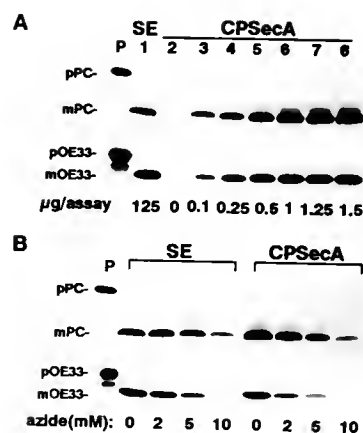


Figure 5-4. CPSecA-supported transport of PC and OE33 is CPSecA concentration dependent (A) and sensitive to inhibition by sodium azide (B). Radiolabeled pPC and pOE33 were assayed for transport into isolated thylakoids in the presence of total stromal protein (SE, 125 $\mu\text{g/assay}$) or varying amounts of CPSecA ($\mu\text{g/assay}$). Sodium azide at concentrations of 2, 5 and 10 mM was included in transport assays (75 μl) supported by SE protein (315 $\mu\text{g/assay}$) or CPSecA (1.2 $\mu\text{g/assay}$) as described in "Materials and Methods". The precursors were pPC from *A. thaliana* and pOE33 from wheat. All assays were 75 μl .

Conclusions

In this study, we have defined one of the pathways for protein transport into thylakoids to be a chloroplast SecA-dependent translocation pathway. Proteins transported on this pathway include PC and OE33, both of which are also present in cyanobacteria, the progenitors of chloroplasts. Suggestive evidence for the operation of a SecA type of translocation mechanism in plant chloroplasts for protein transport into thylakoids was the observation that azide, which specifically inhibits SecA-dependent protein export from bacteria, inhibits PC and OE33 transport but not OE23 and OE17 transport or LHCP integration (Fig. 5-1 in this work; Cline et al., 1993; Knott and Robinson, 1994; Henry et al., 1994). Direct evidence comes from the identification of a SecA homologue from pea chloroplasts (Fig. 5-2) and the demonstration that CPSecA alone is able to support transport of PC and OE33 into isolated thylakoids (Fig. 5-3) in a concentration-dependent and azide-sensitive manner (Fig. 5-4). Our studies are the first to identify and isolate an essential component for chloroplast protein transport *in vitro*.

CHAPTER 6 SUMMARY

The research presented in this dissertation has gone a long way in advancing our understanding of protein targeting and transport into thylakoids. The studies described here made headway in revealing new information and principles about thylakoid protein targeting and transport; they clarified confusions in the literature regarding transport requirements and the role of soluble factors in protein import into chloroplasts and integration into thylakoids; they resulted in the identification and purification of an essential component of the thylakoid translocation machinery.

The key to our success was the use of chemical quantities of purified precursor proteins. With purified precursor proteins, we were able to show that the stromal factor required for LHCP integration plays an active role in the integration reaction, most likely in targeting LHCP to the membrane or in folding LHCP into the bilayer. The use of large amounts of purified precursor proteins additionally allowed us to discover that import of proteins into the chloroplast stroma is much faster than transport of proteins into thylakoids. This unexpected observation allowed us to develop a novel *in organello* competition assay that lead to the discovery of multiple pathways for protein transport into thylakoids.

One of the questions raised by the discovery of multiple pathways was whether transport requirements are characteristic of translocation pathway or of translocated precursor protein. The studies in chapter 5 show that transport requirements are characteristic of translocation pathway and not of translocated precursor protein.

A long unanswered question regarding protein transport into thylakoids was whether chloroplasts use a mechanism for protein transport into thylakoids that is homologous to prokaryotic protein transport. This question was narrowed by the discovery of multiple pathways down to the question of whether any of the three pathways described for protein transport into thylakoids is analogous to the bacterial SecA-dependent translocation system. Our studies were culminated with the identification of a SecA homologue from pea chloroplasts and the demonstration that purified chloroplast SecA is able to promote protein transport into isolated thylakoids.

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BIOGRAPHICAL SKETCH

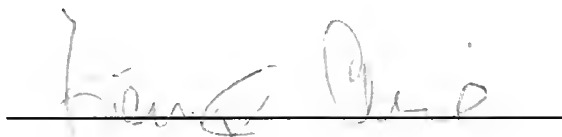
Jianguo Yuan was born on the 15th day of November, 1962, to Mrs. Juxiu Cheng and Mr. Cheng Yuan in Yichun County, Jiangxi Province, the People's Republic of China. Jianguo received his elementary, middle, and high school education from local public school. He went to Jiangxi University in September, 1979, where he received his Bachelor of Science degree in July, 1983, and developed an interest in photosynthesis and chloroplasts. His interest in chloroplast brought him to the Institute of Botany at the Chinese Academy of Sciences in September, 1983, where he worked exclusively on chloroplasts and received a Master of Science degree in October, 1986. Jianguo came to the United States in August, 1988, and has since worked in the laboratory of Dr. Kenneth Cline on the biogenesis of chloroplast proteins.

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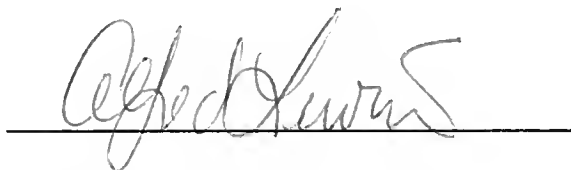
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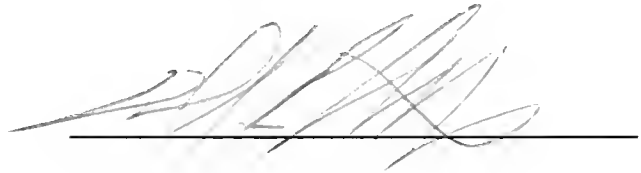
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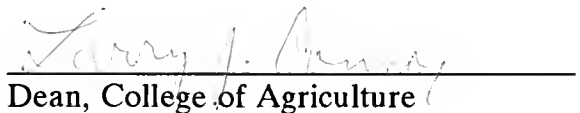
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